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THE DEVELOPMENT AND FUNCTION OF THE ROOT NODULES OF *ALNUS*, *MYRICA* AND *HIPPOPHAË*

by G. BOND, W. W. FLETCHER *) and T. P. FERGUSON **)

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The family Leguminosae provides the classical examples of root nodule plants, and owing to the agricultural importance of that family of plants their root nodules have received continuous investigation for more than 50 years. Though no other large family is similarly characterised by the possession of root nodules, such structures are shown by a number of genera which occur in curiously scattered fashion through the Phanerogams, the only other particular feature in common being that the plants concerned are practically all of woody type. These non-legumes have been the subject of sporadic investigation, chiefly from the standpoint of the identity of the endophytes. Three nodulated genera represented in Europe are those mentioned in the title. These plants are abundant in the appropriate habitats, and the question of the functional significance of their nodules is of ecological and general interest.

The species to which this paper refers are *Alnus glutinosa* (L.) Gaertn., *Myrica gale* L. and *Hippophaë rhamnoides* L. In addition to the function of the nodules, the extent to which their development and activity are affected by environmental factors will also be considered. The comparative treatment of the three species will be based partly on new observations and partly on data already published by the authors in respect of individual species. Aspects which will not be specifically considered include the structure and cytology of the nodules, and the systematic position of the endophytes. In the last connection it may be noted that the endophytes

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have been variously classified as of actinomycetal, fungal, bacterial and myxomycetal nature, this uncertainty being due partly to the difficulty that no confirmed isolation of the nodule organism has so far been achieved.

1. EVIDENCE FOR NITROGEN FIXATION

Previous authors, whose work was reviewed and confirmed by Ferguson and Bond [4]), have reported the successful growth of *Alnus* plants from seed, to a height in some cases of several metres, in a rooting medium free of combined nitrogen, provided nodules were present. The plant begins to show benefit soon after the nodules first develop (Plate IA). Bond [1]) showed that nodulated plants of *Myrica* have a similar capacity for vigorous growth in a nitrogen-free medium, as is further illustrated by Plate IB. The dry weight of this plant, when harvested shortly after being photographed, was 279 g, and the total nitrogen content 5.02 g. In respect of *Hippophaë*, Servettaz [10]) and Roberg [9]) observed that in a nitrogen-free medium nodulated plants grew better than those without nodules. This finding has been confirmed by the present authors (see Plate IC). Nodulated plants as illustrated have attained a nitrogen content of 8 mg, as compared with 0.5 mg for the non-nodulated plants, after 8 weeks' growth in water culture.

It should be noted that in none of the experiments mentioned above were aseptic conditions provided, chiefly because a pure culture of the nodule organism has not been available for inoculation purposes. Other micro-organisms in addition to the endophyte were doubtless present in the rooting medium. The possibility that the fixation of nitrogen that obviously occurred in the experiments was the result of non-nodular activities was examined by Ferguson and Bond [4]) and rejected.

Further evidence that the fixation is a nodular function has been obtained by tests with isotopic nitrogen. These are still proceeding, and here reference will be made to one experiment only. In this the root systems of 3-month-old *Alnus* plants were sealed into glass jars partly filled with culture solution, the air in the space above the solution being then replaced by a mixture of air diluted with an equal volume of nitrogen containing 36 atom per cent of N^{15}. After 6 days' exposure the different parts of the plants were analysed for N^{15} content, with results as shown in Table I.

TABLE I

Test for presence of fixed N^{15} in *Alnus* plants after exposure of the root systems to an atmosphere containing 20 atom per cent excess N^{15} *)

Part of plant	Final N^{15} content, atom per cent	Increase over control
Nodules	0.697	0.329
Roots	0.532	0.169
Tops	0.533	0.170

*) Three plants were exposed to N^{15}, the corresponding parts being bulked together for analysis. Values for N^{15} content of control plants exposed to air only were: — nodules 0.368, roots and tops 0.363 atom per cent.

The enrichment in N^{15} of all parts of the plants confirms that fixation of gaseous nitrogen occurred during the experiment. The finding that the greatest accumulation of N^{15} was in the nodules indicates that these provide the actual site of the fixation. Evidently during the period of the experiment there was considerable transfer of fixed nitrogen from the nodules to the other parts of the plant. These results are in general similar to those reported by Burris, Eppling, Wahlin and Wilson [3]) for legumes.

The present authors' earlier experiments with *Alnus* [4]) and *Myrica* [1]) have shown that after an initial delay the vigour of growth of nodulated plants in nitrogen-free solution is comparable with that of plants supplied with combined nitrogen, as is illustrated for *Myrica* by Plate ID.

2. THE TIME RELATION OF NODULE DEVELOPMENT

The reports of previous investigators have differed as regards the time elapsing between the inoculation of young plants of these non-legumes (with a suspension of crushed nodules) and the appearance of nodules. Krebber [6]) stated that the interval was 6 to 7 weeks in *Alnus* in water culture, a somewhat shorter delay being experienced by Roberg [9]) in *Alnus*, *Hippophaë* and *Elaeagnus*. Hawker and Fraymouth [5]) stated that "Naturally and artificially inoculated seedlings of these (non-legume) plants do not develop nodules until they have reached a considerable size, whereas inoculated clover seedlings develop them soon after germination".

The present authors' experience with plants in nitrogen-free water culture in the greenhouse is as follows. *Alnus* seedlings, inoculated

when 1 to 2 leaves have formed and the shoot is 1.5 cm high, develop nodules in 2 to 3 weeks, in which time the plant has increased only slightly in size. The position is very similar in *Myrica* (Fig. 1). *Hippophaë* seedlings inoculated when the shoots are 2.5 cm high and bear 6 leaves, develop nodules in 16 to 18 days. The inocula employed consisted of suspensions in water of crushed nodules from other plants of the same species, the fresh weight of nodules used per 100 cc of water being 3.5 g for *Myrica* and *Hippophaë*, and 20 g for *Alnus*. Without inoculation these plants have rarely formed nodules under the authors' cultural conditions.

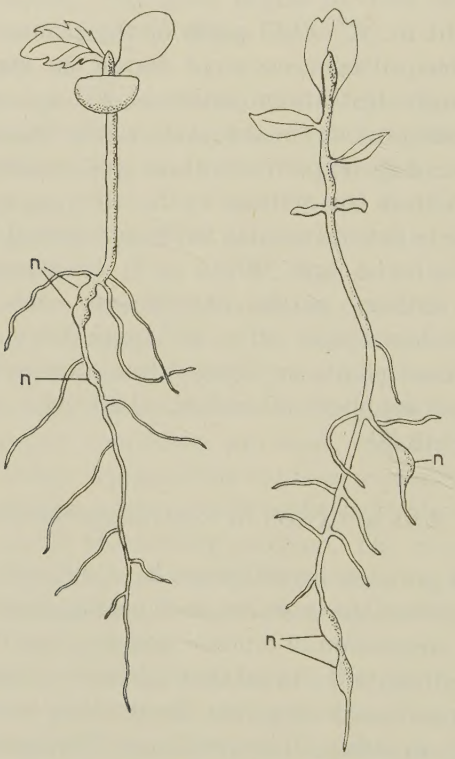

Fig. 1. Young plants of *Alnus* (left) and *Myrica* (right), showing root nodules (n). ($\times\frac{4}{3}$).

By arranging for the endophyte to be in the vicinity of the seed at the time of germination, nodules can be obtained on still younger plants. Thus when *Alnus* seed was sown in inoculated sand, 90 per

cent of the seedlings showed one or more nodules at a stage when only one leaf had emerged from the bud (Fig. 1). A similar result was obtained when seed was sown outside in soil collected from beneath an *Alnus* tree.

Thus under the present authors' conditions the interval elapsing between inoculation and the development of visible nodules is similar in these plants to that in legumes. Also, nodules can be obtained at as young a stage in the development of the plants as in legumes.

3. THE EFFECT OF pH ON NODULE INITIATION

This aspect has been investigated by setting up young plants in Crone's nitrogen-free solution previously adjusted to different pH levels by suitable addition of sulphuric acid or sodium hydroxide, and then inoculating as in the previous section. The results obtained from several experiments are summarised in Table II, data for a typical legume being included for comparison.

TABLE II

Effect of pH of culture solution on nodulation

pH	Percentage of plants forming nodules *)			
	Myrica	*Alnus*	*Hippophaë*	Red clover
3.3	3	0	0	0
4.2	46	45	0	0
5.4	63	77	78	91
6.3	74	81	85	94
7.0	—	89	75	89

*) Each figure is based on the use of at least 35 plants. The data for *Myrica* are taken from Table I of the article by B o n d [1]).

There are two possible effects of the pH of the solution that have chiefly to be considered, firstly that on the nodule organism, and secondly that on the host plant. An adverse effect on either could interfere with nodulation. The relative importance of these can be assessed on the basis of other experiments in which the pH relation of non-nodulated plants supplied with combined nitrogen has been examined. Such plants of *Myrica* [1]) and *Alnus* [4]) grow satisfactorily at pH 3.3, while those of *Hippophaë* and red clover make considerable growth at pH 4.2. These are pH levels at which the corresponding inoculated plants failed to nodulate, except for a small proportion of *Myrica* plants at pH 3.3. Therefore the decreased nodulation in

the more acid solutions is probably to be attributed to the harmful effect of low pH on the nodule organisms. It will be noted that the greatest acid tolerance is shown by the *Myrica* endophyte, followed by *Alnus*, while *Hippophaë* (and clover) show low tolerance.

4. THE EFFECT OF pH ON THE SUBSEQUENT GROWTH OF NODULATED PLANTS

The pH at which nodulation is initially most successful is not necessarily that which produces the best result in the subsequent growth of the plants. Data on this aspect have already been published for *Myrica* [1]) and *Alnus* [4]) with reference to growth during the first season. With *Myrica* best growth was shown at pH 5.4. In a recent larger-scale experiment with *Alnus*, using the same methods as in the already-published work, results were obtained as shown in Table III.

TABLE III

Effect of pH of culture solution of growth of nodulated *Alnus* plants *)

pH	Number of plants harvested	Mean height of shoot in cm	Mean dry weight per plant in g			
			Nodules	Roots	Tops	Total
3.3	0	—	—	—	—	—
4.2	18	49	0.221	0.97	5.56	6.75
5.4	18	52	0.222	0.83	5.36	6.41
6.3	18	43	0.159	0.59	3.92	4.67
7.0	18	37	0.133	0.56	2.72	3.41

*) The plants grew for 5 months in water culture (C r o n e's nitrogen free-solution). All the plants at pH 3.3 failed to nodulate and died. The differences required between means for significance ($P = 0.05$), calculated from analysis of variance, are as follows: — for height of shoot, 5 cm; for total dry weight, 1.05 g.

The statistical treatment indicated below the Table shows that there was no significant difference between the plants at pH's 4.2 and 5.4. The latter were superior to those at pH 6.3, which in turn were superior to those at pH 7.0. It is concluded that the optimum pH for the growth of nodulated *Alnus* plants lies between 5.4 and 4.2. Typical plants from this experiment are shown in Plate IE. The corresponding experiments so far carried out with *Hippophaë* indicate best growth of nodulated plants at pH 6.3.

Thus in *Alnus* the optimum pH for the subsequent growth of nodulated plants is distinctly lower than that for nodule initiation (Section 3), but in *Myrica* and *Hippophaë* there is no clear difference.

In these experiments two pH effects can again be envisaged as likely to operate, firstly an effect on the general growth of the host plant, and secondly a particular effect on the nodule tissues, which may have distinctive pH requirements. Comparable data for non-nodulated plants supplied with combined nitrogen will again assist in distinguishing between these two possible effects. In *Alnus* evidence was obtained that the optimum pH for such plants was 5.4 [4]), *i.e.*, practically the same as that indicated above for nodulated plants. The evidence so far available indicates that the same position holds for *Hippophaë*. In *Myrica*, however, combined-nitrogen plants showed optimal growth at pH 3.3 [1]) as compared with 5.4 for nodulated plants, suggesting that nodule tissues here have a relatively high pH requirement.

Determinations of the actual pH of nodule and root tissues of plants grown in water culture at different pH levels have been carried out. The tissues were crushed in 10 times their weight of distilled water and the pH of the fluid so obtained was determined by meter. Data for *Alnus* are shown in Table IV; rather similar data have been obtained for *Myrica*.

TABLE IV

pH of tissues of *Alnus* plants grown at different pH levels *)		
pH of culture solution	pH of nodules	pH of roots
4.2	5.92	5.02
5.4	5.80	4.93
6.3	6.00	5.21
7.0	6.05	5.15

*) Each tissue pH is a mean of duplicate samples.

It is obvious that the tissues are well-buffered. A tendency for the internal pH to vary in the same direction as the external is just perceptible, but the total variation is slight. It is noteworthy that there is nearly a whole pH unit of a difference between nodule and root pH. Bearing in mind the growth of *Alnus* at different external pH levels (above), it appears that for optimal growth a nodule pH slightly below 6, and a root pH slightly below 5 are required.

5. THE EFFECT OF COMBINED NITROGEN ON NODULATION

A well-known feature of the legume-rhizobium symbiosis is that the extent of nodulation (including both number and size of nodules) is

at a maximum when the plant is grown in a medium free of or very low in available combined nitrogen, and is depressed by the presence of appreciable amounts of nitrate or ammonium-nitrogen. The effect of this factor on the nodulation of non-legumes has been investigated by the following procedure.

Young plants were transferred from sand or peat into water culture in Crone's nitrogen-free solution at a favourable pH, with 5 plants per 2-litre jar. With *Alnus* and *Myrica* different amounts of combined nitrogen in the form of ammonium sulphate were then added to the jars, and the plants inoculated on the following day in the manner described on p. 312. In *Hippophaë*, where owing to rapid root elongation transplanting has to be carried out at a very early stage, all the plants were allowed to grow in water culture for 5 weeks in the presence of 20 mg ammonium-nitrogen per litre, and were then transferred to nitrogen-free solution. Four days later different amounts of ammonium-nitrogen were added to different series of the jars, and inoculation effected the following day. The pH of the culture solution in each jar was checked daily and restored to the original level as necessary. Culture solutions were changed at 2 to 3-weekly intervals and the ammonium-nitrogen dosage repeated. In cultures with low combined nitrogen (5 or 10 mg per litre) the dosage was repeated more frequently, depending on the rate of growth.

The results obtained with *Alnus* are shown in Table V.

TABLE V

Effect of added combined nitrogen on nodulation and growth of *Alnus* plants *)

Mg NH_4-N added per litre of culture soln.	Number of plants harvested	Mean dry weight per plant in g		Nodule weight as percentage of whole plant weight
		Nodules	Whole plant	
0	19	0.008	0.131	6.1
10	19	0.042	1.322	3.2
50	18	0.021	1.734	1.2
100	14	0.016	1.581	1.0

*) Grown for 12 weeks in water culture at pH 6.3.

Plate I

A. *Alnus* plants from seed sown 2 months previously in N-free sand. Nodulated plant on right. ($\times 1/2$).

B. Three-year-old nodulated *Myrica* plant grown from seed in N-free solution. ($\times 1/20$).

C. *Hippophaë* plants in N-free solution, 8 weeks after inoculation, nodulated plant on left. ($\times 1/5$).

D. Five-month-old *Myrica* plants, nodulated in N-free solution on right, non-nodulated supplied with nitrate-nitrogen on left. ($\times 1/6$).

E. Nodulated *Alnus* plants after 5 months' growth in N-free solution of pH (left to right) 4.2, 5.4, 6.3, 7.0. ($\times 1/15$).

Plate I

Plate II

As is customarily observed in short-term experiments of this type, both with legumes and non-legumes, the plants supplied with combined nitrogen made much more growth than those in nitrogen-free solution, since in the latter plants there is an initial delay while nodules form. Thus at harvest the former plants were much larger (see also Plate IIA), and this has to be borne in mind when comparing the extent of nodule development. The Table shows that the absolute dry weight of nodules per plant was greater in all plus-nitrogen series than in the nitrogen-free series (see also Plate IIB and C), this being due more to increased size of the nodule clusters than to an increase in the number of original infection points and eventually of nodule clusters. Relative to the dry weight of the whole plant, however, the dry weight of nodules was highest in the nitrogen-free series, and diminished as combined nitrogen was increased (see right-hand column of the Table). It is concluded that in the presence of combined nitrogen, nodule growth fails to keep pace with the enhanced growth of the rest of the plant. In other words, nodule growth is relatively depressed.

It might be suggested that the lower relative nodule weight was mainly a reflection of the more advanced stage of development attained by the plus-nitrogen plants. While it is probable that even in nitrogen-free solution the relative weight of nodules tends to fall as the plant becomes larger, it should be noted that in Table III plants three times as large as the plus-nitrogen plants in Table V still showed a relative nodule dry weight of 3.4 per cent, confirming that the depression of relative nodule weight in the present experiment was chiefly the result of a specific effect of combined nitrogen on nodule development.

In a comparable experiment with *Myrica* lasting 6 weeks, plants supplied with ammonium-nitrogen were superior in growth, and their nodules also were better developed than in plants in nitrogen-free solution. Dry weights were not taken, but it is probable that the effect of combined nitrogen on nodulation is similar to that in *Alnus*.

Plate II

A. Nodulated *Alnus* plants after 12 weeks' growth, supplied with (left to right) 0, 10, 50, 100 mg NH_4–N per litre. ($\times 1/8$).

B. Experiment as in A. Two plants from 0 mg N series. ($\times 8/7$).

C. Experiment as in A. Root system of plant supplied with 10 mg N. ($\times 8/7$).

D. Root system of *Hippophaë* plant from N-free solution, 5 weeks after inoculation ($\times 4/7$).

The results of a similar experiment with *Hippophaë* are presented in Table VI, and differ markedly from those for *Alnus*. The stronger

TABLE VI

Effect of added combined nitrogen on nodulation and growth of *Hippophaë* plants *)

Mg NH_4-N added per litre of culture soln.	Number of plants harvested	Means per plant			Nodule weight as percentage of whole plant weight
		Number of nodules	Dry weight of nodules in mg	Dry weight of whole plant in mg	
0	22	22	6.5	193	3.4
5	24	5	1.3	283	0.5
10	24	6	1.5	362	0.4
50	18	0	—	619	0
100	19	0	—	684	0

*) Grown in water culture (pH 6.3) for 8 weeks after inoculation.

growth of plants supplied with combined nitrogen was not accompanied by increased absolute weight of nodules. On the contrary this fell sharply, and nodulation was completely suppressed by the presence of 50 mg nitrogen per litre. The few nodules eventually present in the 5 and 10 mg series were late in developing, and it is possible that their appearance was a result of the partial or complete exhaustion of the combined nitrogen at some period. Given a system which ensured the constant presence of 5 or 10 mg nitrogen per litre, it might be that complete suppression of nodules would result, even with such low concentration.

6. GENERAL DISCUSSION

Although evidence pointing to an association of nitrogen fixation with the root nodules of some non-legumes has existed for a long time, it has to a large extent escaped notice. Thus in a recent survey (Whyte, Nilsson-Leissner and Trumble[12]) it is stated, with reference to non-legume nodules, that "their relation to nitrogen nutrition is debatable". In view of the previous and new evidence noted in the present paper, it is clear that the belief that legumes are the only root nodule plants capable of fixing atmospheric nitrogen is no longer justified. Further, the evidence shows that in *Alnus* (and by inference in the other genera) the fixation occurs actually in the nodules. Also it is sufficiently rapid to sustain healthy growth of the plant in a rooting medium devoid of combined nitrogen.

All the evidence so far available relates to cultured plants of the

non-legumes rather than to plants growing in the field, but it would be altogether extraordinary if the faculty for nitrogen fixation existed only under cultural conditions. While it is desirable that direct evidence of fixation in the field should be secured, existing data justify a special ecological importance as nitrogen-providers being assigned to these non-legumes. The fixation associated with *Myrica* and *Alnus* may be of particular significance, since in the acid peat soils favoured by these plants, especially the former (see also below), none of the previously-recognised nitrogen fixing systems is likely to function.

The evidence presented in Section 3 on the differing degrees of acid tolerance shown by the endophytes of these non-legumes under experimental conditions points to the conclusion that the endophytes are not identical. This is in keeping with cross-inoculation studies. Roberg [9] found that *Alnus* and *Hippophaë* were not cross-inoculable, while the present authors have found the same to be true of *Alnus* and *Myrica*. It is perhaps worthwhile pointing out also that the endophytes of *Myrica* and *Alnus* differ sharply from a typical legume organism in acid tolerance (see Table II), and that those non-legumes did not nodulate when inoculated with a mixture of several *Rhizobium* species.

These findings in respect of the acid tolerance of the endophytes are consistent with conditions likely to be existent in the field. In the area studied by the authors, *Myrica* has been found growing in soils of pH's in the range 3.7 to 4.8, and in keeping with this the endophyte showed considerable infective power at pH 4.2, and a little even at pH 3.3. In other parts of Britain *Myrica* occurs in soils of considerably higher pH. *Alnus* plants show a considerable pH tolerance (McVean [7]), but frequently occur in acid regions. Here again the organism showed considerable activity at pH 4.2. Finally, in Britain at least *Hippophaë* is a dune plant growing under conditions of relatively high pH, and in experiment the endophyte was found to be active in media close to neutrality, but to have only slight acid tolerance.

Before considering the effects of combined nitrogen on nodulation (Section 5), it should be emphasized that the question of whether the non-legume nodules continue to fix free nitrogen in the presence of combined nitrogen is an issue not under consideration here. It is being investigated by a different technique.

The reduced nodulation in the presence of combined nitrogen which, as pointed out already, is a well-known feature of legumes, can be regarded as advantageous to the plant (in that metabolic materials are conserved), if it is true that the sole value of nodules to a legume is as a source of fixed nitrogen. The actual causal mechanism has been variously interpreted. The different proposals that have been made were reviewed by Wilson [13]) and are briefly as follows:

(1) that the combined nitrogen exerts a harmful effect on the nodule organism;

(2) that a plant supplied with combined nitrogen, being more vigorous, is more immune to invasion by the nodule organism;

(3) that the early availability of nitrogen makes possible a more rapid synthesis of protein in the plant, thus lowering the level of carbohydrate in the tissues as compared with a plant in nitrogen-free solution, which in turn, it is suggested, may limit the development of nodules. A lower "sink value" appears to be imputed to the developing nodules than to the other parts of the plant. The carbohydrate-nitrogen ratio hypothesis of Wilson (*loc. cit.*) is a further development of this theory.

The relative depression of nodule formation in the presence of combined nitrogen shown to occur in *Alnus* and probably in *Myrica* could conceivably result from the change in metabolic balance visualised in (3), above. The position seems to be different in *Hippophaë*, where the sensitiveness to combined nitrogen not only greatly exceeds that in the two previous genera, but also that typically shown by legumes. The possibility that this result was connected with the fact that all the *Hippophaë* plants had been grown for a period in the presence of combined nitrogen prior to inoculation (p. 316) has been carefully considered. The evidence is against it. Thus the plants which, from the start of the experiment proper, were in nitrogen-free solution, showed the most abundant nodulation yet obtained with *Hippophaë* (see Plate IID), *i.e.*, there was no sign of any interference with nodulation as a result of the pre-treatment with combined nitrogen. It may also be noted that the result of this experiment explains a failure of *Hippophaë* plants to nodulate satisfactorily in several earlier experiments relating to other aspects. It is now clear that this was because small amounts of combined nitrogen had been added at the time of inoculation,

REFERENCES

1) Bond, G., The fixation of nitrogen associated with the root nodules of *Myrica gale* L., with special reference to its pH relation and ecological significance. Ann. Botany (London) N.S. **15**, 447–459 (1951).
2) Bond, G., Some features of root growth in nodulated plants of *Myrica gale* L. Ann. Botany (London), N.S. **16**, 467–475 (1952).
3) Burris, R. H., Eppling, F. J., Wahlin, H. B. and Wilson, P. W., Studies of biological nitrogen fixation with isotopic nitrogen. Soil Sci. Soc. Amer. Proc. **7**, 258–262 (1942).
4) Ferguson, T. P. and Bond, G., Observations on the formation and function of the root nodules of *Alnus glutinosa* (L.) Gaertn. Ann. Botany (London), N.S. **17**, 175–188 (1953).
5) Hawker, L. E. and Fraymouth, J., A re-investigation of the root nodules of species of *Elaeagnus*, *Hippophaë*, *Alnus*, and *Myrica*, with special reference to the morphology and life histories of the causative organisms. J. Gen. Microbiol. **5**, 369–386 (1951).
6) Krebber, O., Untersuchungen über die Wurzelknöllchen der Erle. Arch. Mikrobiol. **3**, 588–608 (1932).
7) McVean, D. N., *Alnus* Mill. J. Ecol. **41**, 447–466 (1953).
8) Miettinen, J. K. and Virtanen, A. I., The free amino acids in the leaves, roots, and root nodules of the alder (*Alnus*). Physiol. Plantarum **5**, 540–557 (1952).
9) Roberg, M., Über den Erreger der Wurzelknöllchen von *Alnus* und den Elaeagnaceen *Elaeagnus* und *Hippophaë*. Jahrb. Wiss. Botan. **79**, 472–492 (1933–34).
10) Servettaz, C., Monographie des Eléagnacées. Botan. Centr. Beih. Abt. 2, **25**, 1–420 (1909).
11) Virtanen, A. I., Biological nitrogen fixation. Ann. Rev. Microbiol. 485–506 (1948).
12) Whyte, R. O., Nilsson-Leissner, G. and Trumble, H. C., Legumes in agriculture. Food and agriculture organisation of the United Nations. Rome. 367 pp. (1953).
13) Wilson, P. W., The biochemistry of symbiotic nitrogen fixation. Madison. 302 pp. (1940).

developed air-spaces than is the case in some legume nodules. On the other hand the non-legume nodules show a superficial layer of cork tissue, but lenticels are present in *Alnus* nodules, while in *Myrica* special negatively-geotropic roots grow from the nodules (B o n d [2]) and are likely to assist in nodule ventilation. It is hoped that further studies now in progress will elucidate some of these aspects.

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SUMMARY

1. It is shown that there is satisfactory evidence of fixation of atmospheric nitrogen by nodulated plants of *Alnus*, *Myrica* and *Hippophaë*. Tests with isotopic nitrogen and other evidence indicate that the fixation occurs in the nodules. The fixation, which is sufficiently extensive to support healthy growth of the plants in rooting media free of combined nitrogen, gives these genera special ecological importance.

2. Under the authors' conditions the interval elapsing between inoculation and the formation of nodules, and also the stage in plant development at which nodules first appear, are similar to those in legumes.

3. As judged by infective power, the acid tolerance of the endophytes of these three non-legumes differ markedly, indicating that the endophytes are not identical. In this respect there is evidence of adaptation to the pH conditions of the normal habitats of the host plants. In *Alnus* the optimum pH for the subsequent growth of nodulated plants is distinctly lower than that for nodule initiation.

4. In a short-term experiment with *Alnus* the presence of ammonium-nitrogen in the culture solution resulted in increased plant and nodule development, but the weight of nodules formed relative to that of the plant as a whole was reduced. The effect of combined nitrogen on *Myrica* was similar. Nodulation in *Hippophaë* proved to be much more sensitive to this factor, since the presence of only 5 mg ammonium-nitrogen per litre greatly reduced nodule development.

5. It is concluded that in functional and developmental respects these non-legume nodules are closely comparable with those of legumes.

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REFERENCES

1) Bond, G., The fixation of nitrogen associated with the root nodules of *Myrica gale* L., with special reference to its pH relation and ecological significance. Ann. Botany (London) N.S. **15**, 447–459 (1951).

2) Bond, G., Some features of root growth in nodulated plants of *Myrica gale* L. Ann. Botany (London), N.S. **16**, 467–475 (1952).

3) Burris, R. H., Eppling, F. J., Wahlin, H. B. and Wilson, P. W., Studies of biological nitrogen fixation with isotopic nitrogen. Soil Sci. Soc. Amer. Proc. **7**, 258–262 (1942).

4) Ferguson, T. P. and Bond, G., Observations on the formation and function of the root nodules of *Alnus glutinosa* (L.) Gaertn. Ann. Botany (London), N.S. **17**, 175–188 (1953).

5) Hawker, L. E. and Fraymouth, J., A re-investigation of the root nodules of species of *Elaeagnus*, *Hippophaë*, *Alnus*, and *Myrica*, with special reference to the morphology and life histories of the causative organisms. J. Gen. Microbiol. **5**, 369–386 (1951).

6) Krebber, O., Untersuchungen über die Wurzelknöllchen der Erle. Arch. Mikrobiol. **3**, 588–608 (1932).

7) McVean, D. N., *Alnus* Mill. J. Ecol. **41**, 447–466 (1953).

8) Miettinen, J. K. and Virtanen, A. I., The free amino acids in the leaves, roots, and root nodules of the alder (*Alnus*). Physiol. Plantarum **5**, 540–557 (1952).

9) Roberg, M., Über den Erreger der Wurzelknöllchen von *Alnus* und den Elaeagnaceen *Elaeagnus* und *Hippophaë*. Jahrb. Wiss. Botan. **79**, 472–492 (1933–34).

10) Servettaz, C., Monographie des Eléagnacées. Botan. Centr. Beih. Abt. 2, **25**, 1–420 (1909).

11) Virtanen, A. I., Biological nitrogen fixation. Ann. Rev. Microbiol. 485–506 (1948).

12) Whyte, R. O., Nilsson-Leissner, G. and Trumble, H. C., Legumes in agriculture. Food and agriculture organisation of the United Nations. Rome. 367 pp. (1953).

13) Wilson, P. W., The biochemistry of symbiotic nitrogen fixation. Madison. 302 pp. (1940).

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OBSERVATIONS ON THE FORMATION AND OXIDATION OF PYRITE IN THE SOIL

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I. INTRODUCTION

It is surprising how long the study of transformation of sulphur in the soil has been neglected. Van Bemmelen [3] [4] started pioneering in this subject in 1863. No important contributions were added to the original work of van Bemmelen until the advent of extensive study of the bottom of the Zuidersea during the enclosure and reclamation of this shallow inlet. One of the first papers resulting from that study was published in 1934 by van der Spek [23], who checked the work of van Bemmelen using more modern analytical methods. A further elaborate study of the soils of the newly reclaimed Zuidersea, including oxidation of sulphides and formation of acid layers, was published in 1936 by Zuur [31]. These studies concerned alluvial marine sediments of the coastal area of the Netherlands. Segeberg [21] in 1937 published his work on acid formation in peat and studied also the role of sulfur in this process while Zörkendörfer [6] some years earlier had analysed the transformations of iron and sulphur in medicinal muds, very rich in minerals, and Rost [19] as early as 1922 reviewed the oldest references about the occurrence of iron sulphides in bogs. Their conclusions confirmed the fundamental study of van Bemmelen.

All the above mentioned communications accepted the interpretation of van Bemmelen about the mechanism of formation and oxidative disintegration of sulphides in soils and muds without a strict proof. The first effort to give this proof and definitely to

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settle the still unsolved problems concerning transformation of sulphur in soils was the work of Verhoop[27]), who in 1940 published her fundamental treatise on chemical and biological transformation of sulphur. Generally the opinion of van Bemmelen was confirmed, which shows how brilliant and advanced his work was. Verhoop's work now allows us to consider the most important points of the mechanism of formation and oxidation of sulphides as sufficiently proved, while the investigations of van Bemmelen, van der Spek and Zuur throw much light on the geology and pedology of the problem.

II. WHAT WAS KNOWN OF THE FORMATION AND OXIDATION OF SULPHIDES AT THE BEGINNING OF THE PRESENT INVESTIGATIONS

Formation of ferrous sulphide and pyrite

Ferrous sulphide (FeS) and pyrite (FeS_2) are so insoluble that they must be considered the end phases of reductive transformation of sulphur in soils and muds (Baas Becking[1])). Consequently both these compounds may be expected in all anaerobic sediments when sufficient reactive iron and sulphur are available, together with any oxygen acceptor to account for the reduction of the sulphur compounds. The iron practically always may be assumed as sufficiently available even for the higher accumulations of sulphides as the very common transformation of ferrous into ferric iron and *v.v.* maintains a great part of the total iron in reactive form. For the origin of the required sulphur two sources are available: sulphates of the sea and brackish waters (sea water contains 0.0825% of sulphur as sulphates (Clarke[5])) and the sulphur in organic compounds. Van Bemmelen[3] [4]) had already developed the theory that sulphides in marine sediments are formed from the sulphates of sea water by reduction under anaerobic conditions when oxidizable organic substances (or elementary iron in the anaerobic corrosion process, von Wolzogen Kühr[28]) [29]), Starkey[25]) [26])) are available. This theory was approved by the extensive work of Beijerink[2]) and others and is now generally accepted. But reduction of sulphates is a strictly endothermic process requiring 118 calories per mol; the realisation of this transformation consequently will be restricted to entirely anaerobic conditions when sulphates represent the sole source of oxygen;

otherwise the disintegration of the proteins of organic matter provides enough hydrogenated sulphur to account for the accumulation of sulphides in most muds and anaerobic sediments. Therefore the formation of FeS and FeS_2 is not bound to the availability of sulphates, nor to marine sediments (C o r r e n s [6])).

The formation of FeS from iron compounds and H_2S, originating from putrefraction or from the reduction of sulphates, can be designated by one of the following schemes:

$$3H_2S + 2Fe(OH)_3 \rightarrow 2FeS + S + 6H_2O \qquad (1)$$

or

$$H_2S + FeCO_3 \rightarrow FeS + H_2O + CO_2 \qquad (2)$$

or when reactive oxygen acceptors are available (*i.e.* organic matter),

$$4H_2S + 4Fe(OH)_3 + CH_2O \rightarrow 4FeS + 11H_2O + CO_2 \qquad (3)$$

It is of no fundamental significance to know which of these three possibilities actually occurs in the sediments, except the formation of elementary sulphur, accounted for by the first mentioned scheme, since this substance was often considered essential for the formation of pyrite according to the scheme:

$$FeS + S \rightarrow FeS_2 \text{ *)} \qquad (4)$$

This last reaction is no longer hypothetical since V e r h o o p [27]) proved it experimentally in coprecipitated, finely dispersed FeS and S in stoppered flasks (even under sterile conditions); but her results were highly erratic, so it appears that this reaction is very sensitive to external conditions. V e r h o o p did not check the reduction potential in her flasks; it might therefore have varied considerably from one flask to another. So, while reaction (*4*) definitely is possible, it must be considered doubtful whether the formation of elementary sulphur under strictly anaerobic conditions may ever be expected as shown in scheme (*1*), since conditions favouring the formation of H_2S will automatically provide enough reactive organic material to reduce the excess of oxygen of $Fe(OH)_3$ and alter scheme (*1*) into scheme (*3*). It seems therefore improbable that pyrite in nature should be formed as designated in scheme (*4*),

*) v a n B e m m e l e n [4]) accepted this way of formation but proposed the following detour: $Na_2S + S \rightarrow Na_2S_2$. In this soluble Na_2S_2 the FeS "dissolved" and finally FeS_2 precipitated from this "solution".

especially since in strictly anaerobic localities elementary sulphur could not always be demonstrated or was found only in small quantities. Consequently another way of formation of pyrite must be presumed. The only logical pathway must be a direct formation either from H_2S and any ferric compound, for instance:

$$8H_2S + 4Fe(OH)_3 + O_2 \rightarrow 4FeS_2 + 14H_2O \qquad (5)$$

or from FeS and H_2S with any hydrogen acceptor:

$$2FeS + 2H_2S + O_2 \rightarrow 2FeS_2 + 2H_2O \qquad (6)$$

These reactions require, besides the highly oxygenated ferric hydroxyde, an additional source of oxygenation. (This additional source of oxygenation need not necessarily be the free atmospheric oxygen; it may even be $Fe(OH)_3$ or any other ferric compound that becomes reduced to the corresponding ferrous compound. Anyhow the soil must contain a surplus of reactive hydrogen acceptors in some form.) Therefore these reactions also do not fit into strictly anaerobic conditions. The formation of pyrite must therefore be considered an oxidation, if H_2S or monosulphides are the presumed starting point, which can be done safely since the formation of H_2S and monosulphides has often been demonstrated as the first step in the putrefaction of sulphur-containing organic compounds and in the reduction of sulphates.

From the above discussion arises the controversy between the numerous statements, that accumulations of pyrite in most cases were found in strictly anaerobic deep strata *), while the formation of this compound must be considered an oxidative process, requiring at least a partial or temporary oxidation of the deposits or the availability of an excess of highly oxygenated substances, such as sulphates, nitrates or ferric hydroxyde. When we discuss our own analytical data in the following pages we will refer to this controversy.

In this connection it must be mentioned that pyrite (FeS_2) is not the only polysulphide of iron. Beside this and the other crystalline form, markasite, different amorphous combinations of Fe and S with polysulphidic properties have been demonstrated. Doss [7]), van der Spek [23]), Zuur [31]), van Bemmelen [4]), Jordis and Schweizer [12]) and Juza and Biltz [13]) have

*) van Bemmelen [4]) therefore accepted as a fact that pyrite is formed when oxygen is absent or nearly so.

studied several of these compounds, and we also found in many soils polysulphides of iron with a variable proportion of Fe and S, but always insoluble in diluted acids.

Whatever the formation of pyrite or any other polysulphides of iron may be, in all investigations these processes were assumed to proceed very slowly. This opinion, originally, was based on the observation that most pyrite in natural sediments was found in deep and old layers. Van Bemmelen [4]) had expressed the opinion that pyrite was formed in the long run in deep anaerobic sediments from FeS and elementary sulphur, and even Verhoop [27]) did not analyse her flasks with FeS and S before $7\frac{1}{2}$ months of incubation at 18°C. But a firm proof for this slowness of the formation of pyrite was never given and Verhoop also accepted it as a fact and did not check it.

Oxidation of ferrous sulphide and pyrite

For a long time the oxidation of ferrous sulphide remained hypothetical though often discussed. It was Verhoop [27]) who studied this process experimentally and succeeded in elucidating it. The earlier investigations presumed the formation of ferrous sulphate as the first product of oxidation:

$$FeS + 2O_2 \rightarrow FeSO_4$$

and consecutively a further oxidation to ferric sulphate and sometimes (by hydrolysis of the latter) to the yellow, insoluble, basic ferric sulphates of varying composition ($3Fe_2O_3.4SO_3$ to $4Fe_2O_3.5SO_3$) so characteristic for clays of marine origin lacking carbonates when drained and aerated — the so-called "cat-clay" (van Bemmelen [4]), van der Spek [23]) [23a]), Zuur [31])). This conception of the process of oxidation, however, was criticized by Verhoop [27]). In clear-cut experiments she was able to demonstrate that no ferrous sulphate was formed, and the first attack on FeS was a strictly chemical splitting of the molecule and an oxidation of the iron to ferric hydroxide while the sulphur accumulated in elementary form:

$$4FeS + 6H_2O + 3O_2 \rightarrow 4Fe(OH)_3 + 4S$$

No enzymatic action of microbes was required for this transformaton. The role of the microbes was in accelerating the otherwise very slow oxidation of sulphur to sulphuric acid and sulphates. The end products of this way of oxidation were the same as drafted by the older

interpretation: $Fe(OH)_3$, ferric sulphates and some free sulphuric acid, resulting sometimes in highly acid "cat-clays".

This assumption, however, does not agree with the experience of Zörkendörfer [30], who in aerated muds found much more ferrous than ferric sulphate, but the oxidation in his case was incomplete with possible secondary reduction of the ferric to the ferrous form. Van der Spek even in his later paper [23a] still refers to the oxidation via $FeSO_4$.

The oxidation of elementary sulphur has been extensively studied from the chemical as well as from the bacteriological point of view by many workers (Waksman, Starkey, Jensen, McIntire, Joffe, Vagler and others), but it is not reviewed here since this process offers no difficulties for the interpretation of our data, which are based on different soils and muds.

For a long time, the oxidation of pyrite remained even more obscure than that of FeS. It was observed by van Bemmelen [4] and later by many other investigators that pyrite oxidation was a much slower process than that of FeS, but the end products were apparently the same and, in soils lacking carbonates, high acidities could be reached and the typical yellow "cat-clays" could be formed. Ohle [16] developed some rather complicated oxidation schemes but presented no reliable experimental proof for his theories, and the paper of Rudolfs [20] in 1922 was not aimed as a complete study of this oxidation process. The first exact experiments on the process of oxidation of pyrite were published in 1952 by Quispel *et al.* [17]. Though these experiments must be considered as preliminary and incomplete, yet they clearly showed that the oxidation of pyrite fundamentally follows the same pattern as that of FeS. Here again an oxidation of the iron to ferric compounds preceeds that of elementary sulphur, formed during the first step of the process, but the pyrite molecule apparently is so much more resistent to attack by atmospheric oxygen that the first splitting requires much more time, and seems also to be accelerated by the activity of sulphur-oxidizing microbes. This stimulation by the sulphur-oxidizing bacteria presumably must be ascribed to the rapid acidification of the substrate, since Quispel *et al.* [17] got a much faster oxidation of pyrite at a low initial pH than at a neutral or alkaline reaction. And since in soils and muds pyrite never occurs as the sole form of reduced sulphur but is always accompanied by other

more reactive substances such as elementary sulphur, labile amorphous polysulphides or FeS, the oxidation of these substances provides the low pH required for rapid oxidation of the pyrite. Therefore under natural conditions a fairly rapid oxidation of pyrite is always garanteed in deposits lacking in carbonates. However even in soils rich in $CaCO_3$, the rate of pyrite oxidation is still fairly high as soon as the aeration is improved.

So it can be stated that, during the extensive pedological work at the Research Department of the Zuidersea Reclamation Authority before and during the last world war, the generally accepted opinion was that pyrite required many years for its formation; consequently accumulations of this compound could only be met in the deeper and older strata of the sediments, while no significant amounts of pyrite could be expected in recently precipitated mud or in particles still suspended in the water. This opinion was so widely accepted that it was not considered necessary to check it. In the same way it was accepted that pyrite, though much more resistent to oxidative disintegration than ferrous sulphide, oxidized fairly rapidly in all soils and muds as soon as they were drained and aerated. This last opinion could be proved by many analyses of soil samples, taken at different depths in the newly reclaimed polders [23] [31] [24]. The bulk of the pyrite, in layers very rich in pyrite, was always found to be oxidized in all the soil down to the level of the water table within a couple of years, even in calcareous soils, where the pH remained neutral or alkaline throughout this whole period; in the upper layers sometimes most of the pyrite became oxidized within two years. However it may not be forgotten that all those analytical results were obtained applying a scheme of analysis in which no especial attempt was made to distinguish between the insoluble sulphates (ferric) and the most resistent fraction of the polysulphides.

III. PRESENT INVESTIGATIONS

The especial interest of the Research Department of the Zuidersea Reclamation Authority in the transformation of sulphur compounds in the soil arose when two very important functions of the sulphur in the newly reclaimed and drained soils were demonstrated: the formation of extremely acid "cat-clay" layers in some parts of the

area and the stabilisation of the structure of the soil after leaching out the sea water electrolytes. Consequently most of the material studied was collected in the reclaimed areas of the previous Zuidersea — the Wieringermeer and the Northeastern Marshes. But for the sake of comparison other soils, of marine origine as well as of other types, were included in the investigation.

During the earlier years of the reclamation of the Zuidersea, fundamental research on sulphur metabolism in the soil permitted a check of the leading theories, whereby the conception, as summarized in the preceding paragraph, was attained. A great handicap in those first years was the lack of sufficiently reliable and accurate but at the same rime rapid, analytical methods, allowing separate determination of sulphates, monosulphides, polysulphides and elementary sulphur in soils and muds. In later years, however, this gap was partly filled by the publication of Smittenberg *et al.* [22]) of adequate methods for the most important analyses of sulphur compounds occurring in soils. This permitted a more extensive study of sulphur transformation in soils and muds to be initiated. At the same time — during the war — many old marshes were inundated or flooded by sea or brackish water and had to be recovered directly after the armistice. So a rich material became available for investigation, concerning not the reclamation of virgin sea bottom but the recovery of old arable land flooded by sea water.

Extensive investigation in the flooded districts resulted in a series of papers ("The inundations in the Netherlands during the second World-War and their effect on the agriculture") of which series the numbers 8, 8*a*, 9 and 9*a* are of most importance in connection with the problems discussed in this paper [8]) [9]) [14]) [15]). All these publications, however — as well as the above mentioned paper of Zuur [31]) — are strictly of the type of applied practical research, not penetrating into fundamental problems. Nevertheless some incidental observations of more fundamental importance were made during that work. It is the aim of this publication to draw the attention to some of these items.

Some observations about the formation of pyrite

For many years, in the Wieringermeer and Northeastern Marshes, only more or less "old" sediments have been investigated. Though geologically some of the soils belonged to the youngest sedimenta-

tions, they nevertheless were deposited many years ago. Their occasionally very high content of polysulphidic sulphur therefore was by no means surprising and fitted very well into the generally accepted scheme of the formation of pyrite in old anaerobic deposits.

During later years, however, when also the really young marine sediments on the tidal mud flats were included in the investigation, often surprisingly high amounts of pyrite were found even in the surface layers,p roved by careful examination, using the methods of Smittenberg[22]), to be really bisulphidic and not elementary sulphur. Thus a much faster formation of pyrite in the mud seemed to be possible than was ever presumed. A complete surprise, however, was the discovery of 0.78% of bisulphidic sulphur in a sample of still suspended marine detritus, accumulating just behind the surfline on the sandy beach near the westpoint of the island of Walcheren. On days with offshore winds the water on the west coast of Holland sometimes becomes turbid and muddy. Such mud, still suspended and constantly agitated in the gentle surf, was collected and immediately preserved and analysed. It proved to contain bisulphidic sulphur, amounting to the above 0.78% of its dry weight, more than was ever found in young sediments on the mud flats or the saltings, but of course still less than can sometimes be found in unaerated deep layers of clay of marine origin with high humus content. In such old soils up to more than 3% of the dry weight can be contributed by sulphidic sulphur (compare the table on p. 38 of the paper of Zuur[31])), and in medicinal muds Zörkendörfer[30]) found as much as 6.5% of sulphur in the dry matter. For illustration, data on some samples of suspended detritus or recently precipitated mud, scraped from the surface of tidal mud flats, are given in table I, together with data on other, but still rather young, deposits.

It is evident from this table that the suspended detritus (samples 1, 2 and 3) and the muds scraped from the flats not later than 48 hours after precipitation on windy days (samples 4, 5 and 6) show no lower pyrite contents than the older sediments represented by the samples 7, 8, 9 and 10, while the former samples scarcely had time for the development of any anaerobiosis. In agreement herewith the detritus samples contain scarcely any monosulphidic sulphur *) and also the day-old sediments had not formed much monosulphides;

*) The traces of monosulphidic S might even have been formed from organic compounds during analysis.

TABLE I

Analytical data of some mud- and detritus-samples (% of dry matter)

No.	Description of sample	$CaCO_3$	Lutum	Humus	Monosulphidic S	Total sulphidic S
1	Suspended detritus, beach Westkapelle	17.7	25.3	n.d.	0.01	0.79
2	Suspended detritus, Balgzand (Waddenzee)	9.5	14.6	3.9	0.03	0.33
3	Suspended detritus, Schiermonnikoog (Waddenzee)	8.6	16.4	n.d.	0.04	0.35
4	Recently sedimented mud, Schiermonnikoog (Waddenzee)	7.6	10.2	2.0	0.16	0.48
5	Recently sedimented mud, N.-Beveland (Kats)	5.9	22.2	5.9	0.13	0.30
6	Recently sedimented mud, Dollard (Carel Coenraadpolder)	11.6	33.8	4.7	n.d.	0.48
7	Salting, south coast of Schiermonnikoog	4.0	18.0	6.3	n.d.	0.04
8	Salting, Dollard (Carel Coenraadpolder)	11.1	42.3	6.9	n.d.	0.25
9	Recently sedimented fresh-water rivermud (IJssel near Kampen)	16.2	14.5	9.2	n.d.	0.09
10	Surface of muddy bottom of the Zuidersea (since the enclosure under fresh water)	10.5	28.1	2.4	0.07	0.27

n.d. = not determined.

some of them had not yet turned black or dark-grey and did not smell of H_2S. It is also clear that the pyrite content does not rise in surface soils after precipitation even under rather anaerobic conditions as prevailed in samples 9 and 10 and partly in 8.

This first indication of the formation of considerable amounts of polysulphides in the detritus of sea water before its precipitation received support from the analyses of numerous samples of mud deposited on old cultivated soil of the island of Walcheren during its inundation in the winter 1944 1945. The tide, through the wide gaps in the dike, had access to the island, eroding deep gullies in some places and forming extensive mud deposits in sheltered areas behind obstacles. This freshly precipitated mud as well as the old cultivated soil of Walcheren have been extensively studied for their content of sulphidic and sulphate sulphur. The old arable land of Walcheren proves to contain scarcely any sulphur *), either before

*) Monosulphides and polysulphides were not determined separately in this investigation.

the inundation, or at the end of a period of flooding of more than a whole year. Only in a small percentage of these samples could higher contents of total sulphidic sulphur than 0.05% on dry weight be found and even then the highest content ever found did not exceed 0.13%. The average content of sulphidic sulphur of such old arable soils in the flooded area could be calculated as *ca* 0.03%, while similar soils of Walcheren and in the rest of the province of Zeeland, when not flooded, contained *ca* 0.01% of sulphidic sulphur. But the recently deposited mud proved to contain much more sulphidic sulphur, averaging *ca* 0.3% of the dry weight, and this figure is definitely too low since many deposits, formed not from fresh sea detritus but from locally transported soil eroded in nearby gullies, have been sampled erroneously as if they were young sea mud deposits. Such samples usually are characterized by coarser granulation, lower water-, $CaCO_3$- and humus contents and also a special colour. Their content of sulphidic sulphur is not higher than that of the parent soils. Such old soil sediments have been as much as possible excluded or treated separately, but in some cases mixed sedimentation with young detritus occurred and then a correct discrimination was very problematic. Anyhow, it can safely be assumed that the young detritus sediments of Walcheren contained more than 0.3% sulphidic sulphur, thus agreeing very well with the figures found in other samples and shown in Table I. Van der Spek in his paper from 1950 [23a]) also mentioned pyrite contents of the upper 3 to 5 cm of mud flats up to 0.48% of dry matter, expressed as S.

All the above analytical data strongly point to a synthesis of polysulphides in marine detritus simultaneously with the formation of the latter, and hence under conditions of rather good aeration. It may perhaps be supposed as a hypothesis, that the formation of a certain amount of bisulphides is inherent to the whole complex process of condensation of soluble compounds of sea water, biogenic elements of plankton and suspended mineral particles in the water to form the flakes of detritus. If this conception is correct, then a certain initial amount of rather stable polysulphides (pyrite) may be a constant characteristic of the detritus of sea water in the shallow muddy coastal waters of the North Sea. Of course, another explanation of the occurrence of pyrite in the suspended detritus of coastal waters can also be considered: the resuspension by tidal currents of

old clay sediments with a high content of pyrite. However, this possibility does not seem to be very probable. The first argument against this explanation is the fact that the pyrite in old sediments is generally accumulated as rather coarse (microscopically visible) spherical pyrite granules, often within the shells of diatoms or in plant residues; such pyrite particles, however, could never be found in still-suspended detritus; besides, the resuspended old clay sediments are generally found in the water as comparatively coarse particles ("klontjes = lumps", see the paper of R e i n h o l d [18]). While some of the samples of suspended detritus, analysed on its pyrite content, contained such clay lumps, the samples with the

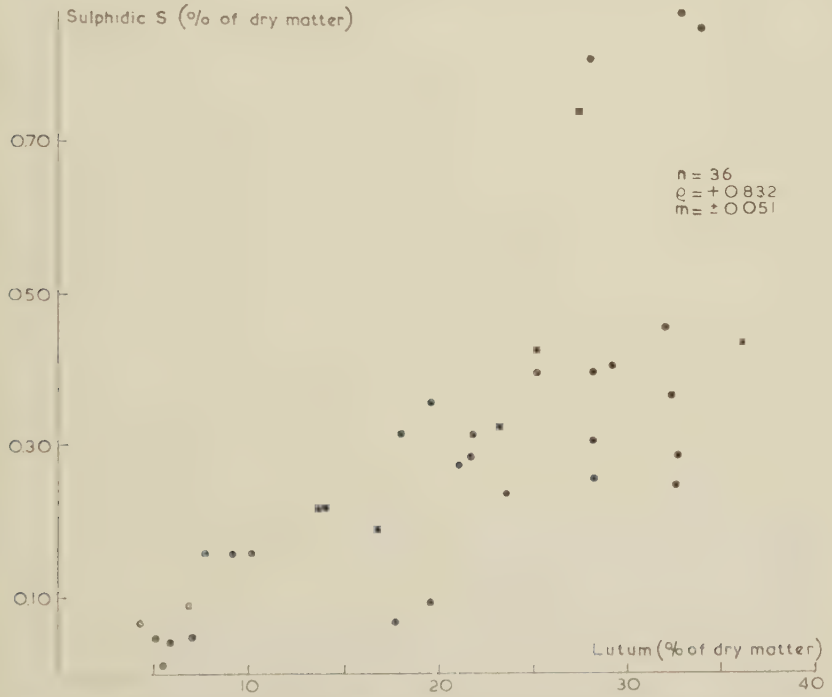

Fig. 1. Relation between sulphidic sulphur and humus in the young marine sediments on the island of Walcheren.

highest percentage of sulphidic sulphur (as *e.g.* No. 1 in Table I) were built up exclusively of the very loose spongy conglomerates of genuine detritis ("vlokjes = flakes"). Another argument is in the observation that the content of sulphidic sulphur correlates rather

well with humus- and lutum contents of the samples. For the numerous analyses of young marine deposits on the island of Walcheren these correlations are shown in graphs Fig. 1 and Fig. 2 and the corresponding correlation coefficients have been found as: $\varrho = + 0.607 \pm 0.122$ for the correlation of sulphidic sulphur and humus and $+ 0.832 \pm 0.059$ for the correlation of sulphidic sulphur and lutum *). These two correlations, however, are not necessarily independent from one another, as is shown by the well known correlation between lutum- and humus contents. In the same

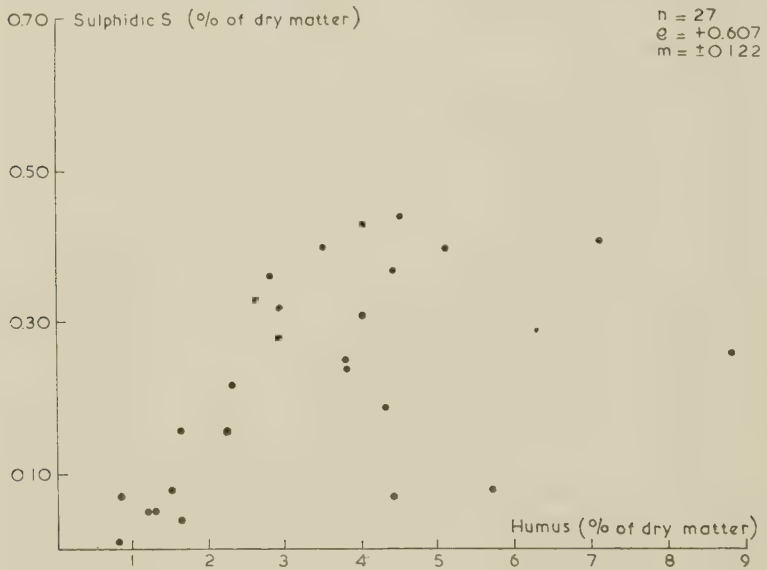

Fig. 2. Relation between sulphidic sulphur and lutum in the young marine sediments on the island of Walcheren.

material this correlation was $\varrho = + 0.786 \pm 0.074$ (see graph Fig. 3). Anyhow the young marine sediments and the detritus are higher in pyrite, the finer they are and the more organic compounds they contain. It would be difficult to coordinate this finding with more or less inclusion of resuspended old deposits with the young sediments.

Thus, while it must be considered possible that part of the pyrite, found in marine detritus originates from old sediments, rich in pyrite, recently resuspended and now incorporated in the detritus, the bulk of the pyrite in the detritus must be a young synthesis,

*) "Lutum" = particles smaller than 2μ.

characteristic for the formation of detritus. This assumption of accumulation of pyrite under fundamentally aerobic conditions — though rather incredible when viewed superficially — might perhaps be in agreement with the fact, discussed already on p. 327, where the formation of pyrite was recognized as not a strictly anaerobic process. The formation of some labile sulphidic compounds, if need be restricted to some larger plankton organisms, is still a rather

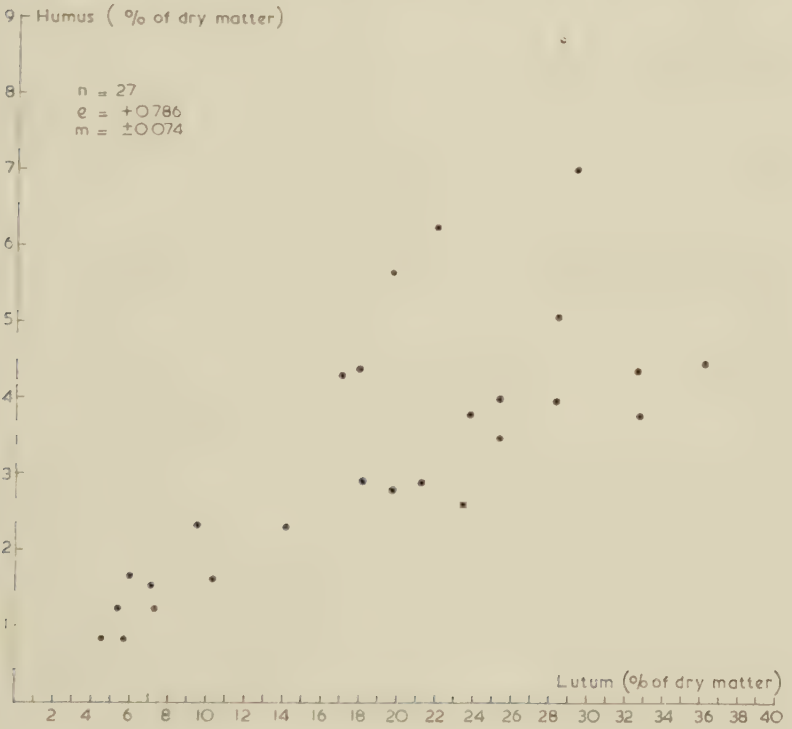

Fig. 3. Relation between lutum and humus in the young marine sediments on the island of Walcheren.

doubtful presupposition, while the precipitation of the extremely insoluble pyrite from that presumed sulphidic compound and $Fe(OH)_2$ seems not to meet any opposition. It is also probable that formation of stable pyrite in suspended detritus is only possible because of the presence of some mineral clay particles, since this part of the material may provide for the necessary iron compounds and may serve as an adsorption complex for the polysulphides.

If the above proposed hypothesis would turn out to be correct, then all marine muddy sediments must have been precipitated with a certain initial amount of pyrite, generally amounting to some 0.3 to 0.5% on the dry matter, expressed as sulphur. The highest content on record was 0.79%. This rather significant content, however, does not explain the much higher percentages of pyrite often found in old sediments, as they have been studied by van Bemmelen [3) 4)], van der Spek [23)], Zuur [31)], Zörkendörfer [30)] and others. It seems impossible to ascribe those high concentrations of pyrite also to a synthesis during precipitation. A consecutive accumulation of pyrite from soluble sulphur compounds (SO_4^{--} of the sea water, or H_2S from the disintegrating organic matter) and any source of Fe-ions on account of the oxidation of oxygen acceptors, as has been more than once proposed, may be an explanation.

An indirect support for this conception is the very clear-cut parallellism between the amount of oxidizable organic matter in a deposit and its pyrite content. In the Wieringermeer Marsh the samples with the highest pyrite content were also very high in humus. Such "peaty" layers could often be found in the heavy clay deposits in those parts of that marsh where, during the sedimentation, dense vegetation of reeds or of halophytes prevailed. Sample A in the table on p. 38 of the paper of Zuur [31)] is an example of such a peaty clay soil with 3.65% sulphur on the dry matter, while other samples with normal humus contents never surmounted 1.5 to 2.0% of sulphur. Van Bemmelen [3) 4)] and van der Spek [23)] also underlined the correlation of humus- and pyrite contents. In some cases the high amount of available humus disappeared after the formation of the deposit, but some characteristic plant residues still show that originally much oxidisable organic matter was available for the accumulation of sulphides.

A much more direct proof for the accumulation of sulphides and especially of pyrite in deposits after their precipitation could be detected from the formation of pyrite in flooded soils in Zeeland with an extraordinarily high amount of fresh plant residues. On p. 334 the general observation was expressed, that the old cultivated soils of Walcheren had not formed considerable amounts of sulphides during the period of flooding, though a small increase from the average of *ca* 0.01% of sulphidic sulphur in ordinary unflooded land to the average of *ca* 0.03% after a year of flooding with only inci-

dentally higher amounts up to 0.13% had been demonstrated. This general observation, however, holds only for arable land, usually low in fresh organic matter, while the grasslands had accumulated much higher amounts of pyrite. Unflooded grassland in Zeeland had the same very low content of sulphides as arable land, *i.e. ca* 0.02% of sulphidic sulphur, but at the end of the year of flooding this percentage had risen to the average of *ca* 0.12% varying between 0.04 and 0.23%. A similar result could also be found in soils flooded for more than one year. Such soils were available in small previously cultivated polders, in later years flooded and abandoned ("Dijkvallen" or "Inlagen"). Some examples of such soils are collected in Table II.

TABLE II

Analytical data of some flooded old cultivated soils (% of dry matter)

No.	Description of the samples	$CaCO_3$	Lutum	Humus	Mono-sulphidic S	Total sulphidic S
1	Bohemenpolder, 13 years of flooding, arable land, 0–10 cm	7.5	18.0	1.6	0.02	0.02
2	The same, 10–20 cm	10.7	23.2	1.1	0.02	0.02
3	Oost-Bevelandpolder, 6 years of flooding, arable land, 0–20 cm .	8.9	24.0	1.9	0.00	0.01
4	Sas van Goes, 3 years of flooding, arable land, 0–20 cm	6.2	13.0	2.5	0.00	0.03
5	The same, another plot with more humus	4.8	11.7	5.1	0.01	0.01
6	Bohemenpolder, 13 years of flooding, grassland, 0–10 cm, sandy soil . .	4.4	8.4	3.0	0.10	0.15
7	The same, 10–20 cm	5.4	8.1	2.2	0.06	0.11
8	Leendert Abrahamspolder, 4 years of flooding, grassland, 0–5 cm . . .	0.5	25.6	13.5	0.13	0.22
9	The same, 5–20 cm	1.2	31.5	7.7	0.04	0.11

These figures clearly demonstrate a significant — though very slow — formation of mono-sulphides and also pyrite in the grassland samples 6–9, while the samples from the former arable land (1–5) scarcely show any sulphides. In all the flooded grasslands only the thin turf served as source of organic matter. But in thick vegetation of reeds or halophytes much more organic matter is formed and, besides, flooded grasslands do not form year by year new organic matter; they are destroyed by the flooding, while natural halophytic vegetation thrives in the brackish water and produces throughout

many years great amounts of organic matter. Such conditions must be considered ideal for an accumulation of sulphides up to the high concentrations reported by van Bemmelen, van der Spek, Zuur and also found during the present investigation in old marine sediments.

Summarizing, it can therefore be said, that the above analytical data seem to point to two fundamentally different ways of pyrite formation: *1*. Directly during the formation and sedimentation of the detritus in the sea and in the estuaries, and *2*. in older sediments if rich in humus or any form of organic matter especially in heavy deposits covered by a dense vegetation, which provides every year for fresh organic matter. The first way of formation holds for all marine sediments — in the tidal zone as well as in deeper water; the second is practically restricted to tidal land with a dense vegetation, thus to brackish inlets or estuaries.

No effort has been made so far to study the chemical properties of the pyrites formed in these two ways. It is not improbable that these substances should prove to be fundamentally different, though both are polysulphides of iron. The proportion of Fe and S in the polysulphides (insoluble in concentrated HCl but soluble in aqua regia or reducible with Sn [22]), offered no clear-cut indications for a significant difference between the pyrite in suspended detritus and in old bluish anaerobic clay soils. In both cases a rather significant surplus of S was found, varying between 2.2 and 3.5 against 1 part of Fe. The most resistent part of the polysulphides consequently seems not to be pure pyrite but either a hypothetical polysulphidic compound with more S than corresponds with the formula FeS_2 or a mixture of iron bisulphide with bisulphides of other cations, as insoluble in HCl as pyrite.

Some observations about the oxidation of pyrite

Though slow, if compared with that of iron monosulphide, the oxidation of pyrite has always been considered a rather fast process. Consequently no pyrite was expected in the well aerated ploughed upper layers of cultivated soil. This generally accepted opinion was confirmed by analytical data collected during the extensive investigation in Zeeland after the inundations of the last war. Applying the technique of Smittenberg [22] in unflooded soils, on arable plots as well as on grasslands, significant amounts of pyrite could

never be found; the total sulphidic sulphur content seldom surpassed 0.03% calculated on dry soil. Consequently the impression was received that these observations were in agreement with the older experience mentioned on p. 330. It was therefore really a surprise in 1946 to find 0.4% polysulphidic sulphur in a sample of a marine clay soil from the Wieringermeer Marsh. This sample was collected in 1937 for an experiment on humus disintegration. About 0.25 m^3 of soil from the upper 20 cm was carefully crumbled, sieved and put in a big unglazed earthenware container, where the soil was kept under optimal conditions of aeration and temperature from 1937 onward. The conditions of moisture and aeration were regularly checked. The whole mass of the soil more than once has been taken out of the container and mixed; never has any surplus of moisture been detected near the bottom of the container. Though at the time of collecting the sample in 1937 this soil was still a very young one, since the Wieringermeer Marsh was reclaimed in 1930 and the drainage of the plot, where the sample has been taken, was not operating before 1932, from the moment of sampling the soil in the container was kept under the conditions of optimal aeration. So, all sulphides during a period of 9 years were exposed to the most vigorous oxidation, and it could not be expected that this soil in 1946 should still contain a significant amount of sulphidic sulphur. Yet, 0.4% was found on analysis. This unexpected result was sufficient reason for a more extensive investigation of many clay soils in different areas of the Netherlands. Some representative analyses are shown in Table III.

Samples 1, 2, 3 and 4 were collected in the relatively young Wieringermeer Marsh, reclaimed in 1932 (sampled in 1946), but with a perfect modern drainage system. All of them, except No. 3, which represents a very light sandy soil, proved to contain considerable sulphidic sulphur (no monosulphides soluble in HCl were found in any of the soils shown in Table III), so the first preliminary impression was that a certain fraction of the pyrite in soils, originally rich in pyrite, is oxidized very slowly, remaining still unchanged even after 14 years of drainage and cultivation. The next analysed soil was No. 5 from the Groetpolder, a marsh reclaimed in 1844. Here 0.19% of polysulphidic sulphur was still found, notwithstanding the fact that this soil was drained and cultivated as arable land for more than 100 years. Now a series of comparable marshes, consecutively enclosed

and drained during the last centuries up to recent years was selected near the Dollard inlet in the province of Groningen. Samples 6, 7 and 8 represent ages varying from 26 to 280 years. Here none of the soils proved to contain appreciable amounts of sulphides, a finding in line with the soils in the province of Zeeland (in Table III represented by No. 13). So it seemed as if the Wieringermeer and the adjacent

TABLE III

Analytical data of some well aerated old cultivated soils

No.	Description of the samples	$CaCO_3$	Lutum	Humus	Total sulphidic S	Reclaimed in
1	Sample in container, aerated since 1937, Wieringermeer	14.2	26.9	3.2	0.40	—
2	Wieringermeer, lot O 36, the same as where No. 1 is taken	16.5	21.1	3.4	0.36	1932
3	Wieringermeer, lot H 2, light sandy loam	12.5	3.8	1.0	0.04	1932
4	Wieringermeer, lot D 65, heavy clay soil	14.0	21.6	2.9	0.27	1932
5	Groetpolder, heavy clay soil.	7.1	41.2	4.7	0.19	1844
6	Reiderwolderpolder, heavy clay soil	9.7	41.2	3.3	0.02	1920
7	Oosterwolderpolder, heavy clay soil	4.0	45.7	3.5	0.01	1769
8	Oud-Nieuwlandpolder, heavy clay soil	0.1	46.0	3.6	0.00	1665
9	Kampen, fresh-water deposit of the river IJssel, sandy loam	—	—	—	0.00	very old
10	Kampen, fresh-water deposit of the river IJssel, heavy old garden soil	—	—	—	0.01	very old
11	Kesteren, fresh-water deposit of the river Rhine, heavy clay soil outside of the dike, flooded sometimes in winter	—	—	—	0.21	—
12	Lienden, fresh-water deposit of the river Rhine, heavy clay soil, old cultivated arable land	—	—	—	0.19	very old
13	Walcheren, marine light clay soil, not flooded	1.7	9.9	8.0	0.02	very old

marsh of the Groetpolder occupied an exceptional position. But soon even this restriction of the problem became untenable when not only among marine sediments, but also among river clay deposits some representative samples were found to contain rather high amounts of pyrite, as demonstrated by No. 11 and No. 12 of Table III. Especially No. 12 is illustrative as this soil has been under normal drainage and cultivation from times immemorial. The occurrance of pyrite

in old cultivated soils became even more erratic when some other river-clay deposits (Nos. 9 and 10, the latter being a very heavy and poorly drained soil rich in organic matter), proved not to contain any pyrite, though they were situated close to the sea in the estuary region, contrary to the Nos. 11 and 12, originating from more eastern localities far away from the sea.

Another spectacular demonstration of polysulphides practically unharmed by ordinary oxidation in well aerated soils was found in the second marsh, reclaimed in the former Zuidersea, the Northeastern Polder. Here, the sulphur content of some experimental plots was determined regularly during a series of years. Alterations of the sulphur content could therefore be followed. These analyses were not performed by the methods developed by Smittenberg *et al.* [22], but by the classical methods. Only the amount of total sulphur (the aqua-regia extraction) and of water-soluble sulphur (sulphates) was determined, but after the period of oxidation the water-soluble sulphur content became very low (0.01% or less) while the "total sulphur" proved neither to be any form of elementary sulphur nor the water-insoluble basic ferric sulphate (= "cat-clay"), as these soils were rich in $CaCO_3$ and had a pH between 7.5 and 8.0, and besides, consecutive determination by the Sn-reduction method of Smittenberg [22] showed that the bulk of the sulphur in these soils was still polysulphidic. Unfortunately in none of these cases was the original soil very rich in sulphides. The total sulphur content varied between 0.4 and 0.6% of the dry soil. Yet the analyses demonstrated that only part of the water-insoluble sulphur became oxidized during the first one or two years, while thereafter no further decrease of the insoluble sulphur could be shown: this content became stabilized on a level of some 0.3% to 0.4%, as shown in Fig. 4. The amount of water-soluble sulphur in these soils remained rather high during the period of oxidation of the sulphides, but soon thereafter it fell to the insignificant figure of less than 0.01%. In the soils of the Northeastern Polder, consequently, the same situation was found as in the Wieringermeer, notwithstanding the fact that the upper layers of the latter were sedimented thousands of years ago, while in the Northeastern Polder the upper layers were deposited during recent decades or the last 100 years. Considerable amounts of polysulphidic sulphur (up to 0.1%) have also been reported by Van der Spek [23a] in some well aerated soils in the Haarlemmer-

meer and near Zevenhoven in marshes reclaimed already long ago.

It is still impossible to give any reasonable explanation for this high resistance of some fractions of the pyrite in some soils. It could only be demonstrated beyond any doubt that some old and well aerated soils contained significant percentages of pyrite, while other soils were entirely free from pyrite. No rule for this difference could be detected. It is, however, evident that the iron polysulphides in soils must be represented by different fractions: more and less resistent against oxidation by atmospheric oxygen. Sharp disparity in this respect suggests the existence of a small number of entirely different types of iron polysulphides: readily oxidisable and stable with few intermediate forms. But this hypothesis had still to be checked, since the stability of some types of iron polysulphides could be questioned.

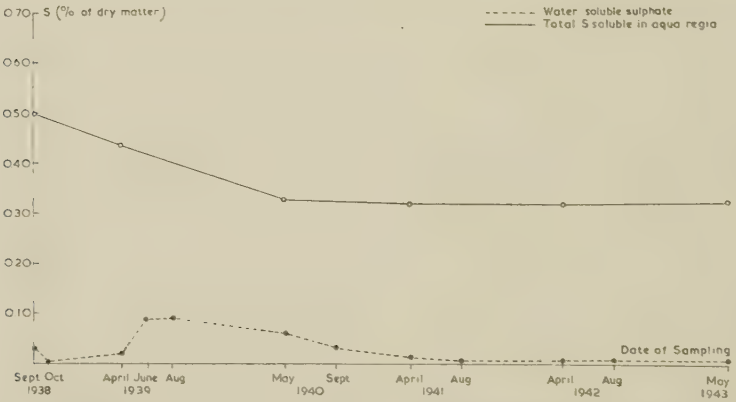

Fig. 4. Oxidation of pyrite in a clay soil of the Northeastern Marsh during the first years after reclamation.

With the object of gaining insight into the composition of total polysulphides in marine deposits and to distinguish between fractions with different resistances against oxidation, a still unaerate bluish clay from deeper layers of the soil in the Wieringermeer Marsh was dispersed and treated with different concentrations of hydrogen peroxide under standardized conditions: 2 g of air-dry ground soil were dispersed in distilled water and agitated during 6 hours, for a complete dispersion of all sulphides; the required amount of H_2O_2 was added, the suspension boiled for one minute, rapidly cooled, and filtered. In the filtrate, sulphate was determined by the colorimetric method with benzidine [15] [18].

The amounts of sulphur extracted from the soil, expressed as % of the dry soil are shown in Fig. 5 plotted against the concentration of H_2O_2 used. The sulphates in the original soil sample before any oxidation are subtracted from all analyses, consequently the figures shown in the graph represent exclusively the sulphate derived by oxidation from sulphidic sulphur. The original sample contained a total amount of 0.71% sulphidic sulphur and Ca CO_3 in excess, while no elementary sulphur was present.

On analysis the curve drafted through the individual determinations proved to correspond closely with a logarithmic function, suggesting a rather regularly mixed composition of the polysulphides comprising all grades of resistance against oxidation. No support for the existence of only a few fundamentally different forms of polysulphides thus could be derived from these experiments.

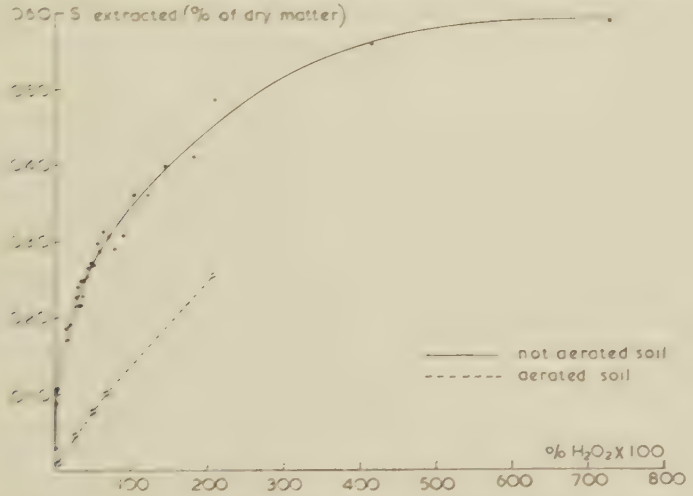

Fig. 5. Oxidation of polysulphides by H_2O_2 in aerated and not aerated soils from the Wieringermeer Marsh.

In Fig. 5 is also shown the same graph for the well aerated soil in 1937 collected in the same area of the Wieringermeer Marsh (No. 1 in Table III). In this soil all easily oxidizable fractions of the polysulphides were already transformed, therefore only the most stable form remained in the sample. As a consequence the curve for this soil rises less steeply, but even rather weak concentrations of H_2O_2 extracted significant percentages of the total sulphides. And again

a smooth curve was found with no indication of the existence of sharply separated forms of polysulphides.

Similar experiments using nitric acid instead of H_2O_2 had the same negative result. Therefore no acceptable explanation for the occurrence of pyrite in some soils after many years of optimal aeration could be found. Future investigation will be needed to solve this problem. Now in this connection only the attention must be drawn to the exceptionnally high S : Fe proportion in the stable phase of pyrite in the old aerated soils. While in unaerated old deposits (the blue clays) and also in the young sediments of marine detritus the S : Fe proportion in the polysulphides was found to vary between 2 : 1 and 3 : 1, this proportion in the aerated old pyrite containing soils exceeded 3 : 1 and in some samples was as high as nearly 4 : 1. Any factors retarding the oxidation of sulphur in the polysulphides must be responsible, while part of the iron has already been extracted or transformed to the very insoluble iron phosphate [17]).

The discovery of old arable soils with comparatively high amounts of pyrite originally was felt as a threat to the practical work on the flooded soils in Zeeland in 1946, where the high pyrite content was used as an indication of young sediments or of flooded grasslands, whereupon no treatment with $CaSO_4$ was needed for the recovery of the structure [8]) [15]). But fortunately no old cultivated soils with high pyrite figures were ever found in Zeeland, either flooded or unflooded, except the turf layer of flooded old grasslands. Consequently within that area the quantitative determination of pyrite could be used for mapping the plots requiring $CaSO_4$ application, but this validity can not be claimed for other soils in other regions.

SUMMARY

A critical review of the present conception of the transformation of sulphur compounds in soils and muds is given, to serve as introduction to the present investigations. Some of these generally accepted ideas are criticized:

1. The formation of polysulphides of iron was shown to be possible not only under strictly anaerobic conditions, as a slow process, resulting in an accumulation of pyrite on the long run in deeper strata of waterlogged soils, but also very rapidly in the detritus, while still suspended in sea water. It even seems to be questionable whether the bulk of the pyrite found in deep layers of marine sediments really was synthesised so far below the

surface of the soil. It can also be presumed that the formation of pyrite was completed when these layers still represented the surface of saltings or reed-covered estuary banks. It is not certain whether the two types of pyrite formed under fundamentally different conditions are the same substance or represent different types of iron polysulphides.

2. The generally accepted idea that pyrite can readily be oxidized, when exposed to a free access of the atmosphere, also proved to be incorrect for some soils. In some areas of Holland, even in very old arable land with a perfect drainage system, appreciable amounts of pyrite could still be demonstrated. This surprising resistance of part of the pyrite in such soils presumably must be ascribed to different proportions of phosphates and pyrite in those soils. The fundamental effect of phosphates on the oxidizability of pyrite has already been demonstrated by Quispel *et al.* [17]).

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REFERENCES

1) Baas Becking, L. G. M., Chem. Weekblad **35**, 502 (1938).
2) Beijerinck, M. W., Centralbl. Bakteriol. Parasitenk. II **1**, 1; 49; 104 (1895).
3) Bemmelen, J. M. van, "Bouwstoffen tot de kennis van de kleigronden der provincie Groningen"; G. J. Mulder, Scheik. Verh. en Onderz. Deel III, stuk 2, 1 (1863).
4) Bemmelen, J. M. van, "Bijdrage tot de kennis van den alluvialen bodem in Nederland"; derde verhandeling ("De samenstelling en vorming van de zure gronden in het Nederlandsche alluvium"); Verhandel. Koninkl. Akad. Wetenschap. Amsterdam, Afdeel. Natuurk. **25**, 33 (1886).
5) Clarke, F. W., "Data of geochemistry"; Washington, U.S. Geol. Survey Bull. 770, 5th ed. (1925).
6) Correns, C. W., "Wissenschaftliche Ergebnisse der Deutschen Atlantischen Expedition 1925–1927". 1e Lieferung, Berlin (1935).
7) Doss, B., Neues Jahrb. Mineral. Geol. Beilage Bd. **33**, 662 (1912).
8) Harmsen, G. W., No. 8 of: De inundaties in Nederland gedurende de tweede wereldoorlog en de gevolgen daarvan voor de landbouw. "Microbiologische processen in geïnundeerde gronden tijdens en na de overstroming". I. (In press).
9) Harmsen, G. W., No. 8*a* of: De inundaties in Nederland gedurende de tweede wereldoorlog en de gevolgen daarvan voor de landbouw. "Microbiologische processen in geïnundeerde gronden, tijdens en na de overstroming". I. (In press).
10) Hofstee, J. and Timmer, H. J., "Analysemethoden voor grond, gewas, water en bodemvocht". Mimeographed report from the Research Department of the Zuidersea Reclamation Authority, Kampen (1952).
11) Jones, D. G. and Wood, C. E., Z. Anal. Chem. **98**, 456 (1934).
12) Jordis, E. and Schweizer, E., Z. Anorg. Chem. **23**, 577 (1910).
13) Juza, R. and Biltz, W., Z. Anorg. Allgem. Chem. **205**, 273 (1932).
14) Molen, W. H. van der, No. 9 of: De inundaties in Nederland gedurende de tweede wereldoorlog en de gevolgen daarvan voor de landbouw. "De kationenhuishouding in met zeewater geïnundeerde gronden". (In press).

15) Molen, W. H. van der, No. 9*a* of: De inundaties in Nederland gedurende de tweede wereldoorlog en de gevolgen daarvan voor de landbouw. "verval en Herstel van bodemstructuur in de inundatiegebieden". (In press).
16) Ohle, W., Arch. Hydrobiol. **30**, 604 (1936).
17) Quispel, A., Harmsen, G. W. and Otzen, D., Plant and Soil **4**, 43 (1952).
18) Reinhold, Th., Mededel. Geol. Stichting, N.S. No. 3, 1948–1949, p. 75 (1949).
19) Rost, C. O., Soil Sci. **14**, 167 (1922).
20) Rudolfs, W., Soil Sci. **14**, 135 (1922).
21) Segeberg, H., Bodenkunde u. Pflanzenernähr. **4**, (49); 50 (1937).
22) Smittenberg, J., Harmsen, G. W., Quispel, A. and Otzen, D., Plant and Soil **3**, 353 (1951).
23) Spek, Jac. van der, "Bijdrage tot de kennis van de zure gronden in het Nederlandsche Alluvium"; Verslag. Landbouwk. Onderzoek., **40** B, 409 (1934).
23a) Spek, Jac. van der, „Katteklei"; Verslag. Landbouwk. Onderzoek. **56.2**, 1 (1950).
24) Spithorst, C., Mimeographed report of the Research Department of the Zuidersea Reclamation Authority (1932).
25) Starkey, R. L., Antonie van Leeuwenhoek **12**, 193 (1947).
26) Starkey, R. L. and Wright, K. M., "Anaerobic corrosion of iron in soil"; Bull. Techn. Sect. Am. Gas Assoc. New York (1945).
27) Verhoop, J. A. D., "Chemische en Microbiologische omzettingen van ijzersulfiden in den bodem", Thesis, Univ. Leiden (1940).
28) Wolzogen Kühr, C. A. H. von, Water **22**, 33; 45 (1938).
29) Wolzogen Kühr, C. A. H. von en Vlugt, I. S. van der, Water **18**, 147 (1934).
30) Zörkendörfer, W., Balneologe **2**, 387; 461 (1935).
31) Zuur, A. J., "Over de Bodemkundige Gesteldheid van de Wieringermeer", Dep. of Communication, The Hague (1936).

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BORON, AN ESSENTIAL MICRO-ELEMENT FOR *AZOTOBACTER CHROOCOCCUM*

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INTRODUCTION

Though for the determination of boron in soils more or less satisfactory chemical methods are available it seemed worthwhile to investigate the possibility of a microbiological determination as well.

There is as yet but little known about the necessity of boron for the growth of micro-organisms. The French investigator V o i c u [14]) was the first to report of a stimulating effect of boron on *Azotobacter*. In 1922 he published a study on the influence of humus on the sensitiveness of *Azotobacter chroococcum* towards boron; the quantity of nitrogen fixed by the *Azotobacter* increased eightfold by the addition of 0.1% sodium humate only. Adding moreover 1 10 ppm boron, N fixation increased with 5.7%. Curiously the toxic effect of 25–100 ppm boron was much greater in the presence of humate than in its absence.

According to H e r z i n g e r [6]), the growth of Algae is favourably influenced by the addition of small amounts of boron. A culture solution with 10 ppm boron was optimal for *Chlorella chlamydomonas* and *Chlorococcum humicolum*. The present authors, however, failed to observe any favourable influence of boron on *Chlorella*, whereas 10 ppm decidedly reduced the growth of this organism. E y s t e r [4]) working with *Nostoc muscorum* showed that this organism needs 0.1 ppm boron for optimal growth, whereas concentrations greater then 1 ppm were toxic.

H e r z i n g e r also noticed that the development of *Azotobacter*, nitrifying bacteria and a number of anaerobic bacteria increased

by addition of small amounts of boron, in a concentration of about 0.1 ppm. In his paper, however, quantitative data are missing and no details are given of the ways, in which the chemicals were purified.

Hanna and Purvis[5]) observed an increase in CO_2 liberation of 8.7–9.9% from Norfolk sandy loam, pH 5.1, to which dextrose was added, at the addition of 10 ppm boron as borax. When the pH was raised to 7.1 by liming, the addition of 10 ppm boron caused an increase of 3.3–20.3% in CO_2 liberation.

In 1948 miss Jogeswari[7]) showed that *Fusarium vasinfectans* produced three times as much mycelium with 0.3 ppm boron as without boron.

Experiments carried out in the author's laboratory showed that the growth of *Fusarium vasinfectans*, however, was too slow to make the use of this mold attractive for practical soil investigation.

Edson, Novak and Smith[3]) published the results of a preliminary study of a microbial test for available boron in soils, using CO_2 production by *Penicillium chrysogenum* as an indicator. A test can be made with only one gram of soil, which seems to be convenient for the practical determination of boron in soil samples.

Though it is a well established fact that nitrogen fixation by the root nodules of leguminous plants is closely related to an adequate boron supply, the authors failed to show any influence of boron on the development of *Rhizobium*, *in vitro*, even with the same purified chemicals and aluminium apparatus, which were successful in the case of *Azotobacter*.

In 1950 Jordan and Anderson[8]) published a paper on the effect of boron on nitrogen fixation by *Azotobacter*. Though their experiments indicate that boron stimulates the growth of *Azotobacter*, their results are somewhat conflicting. When they cultivate *Azotobacter* during 7 days they find an optimum at 3 ppm, whereas in a 3-week old culture there is a minimum at 3 ppm and a optimum at 20 ppm.

Accordingly they are guarded in their conclusion, saying that the boric acid treatment in pure cultures *seemed* to increase nitrogen fixation by *Azotobacter chroococcum*.

As so little is positively known about the influence of boron on the growth of micro-organisms, it seemed worthwhile to investigate this problem more in detail. The present investigations were

started with a boron-deficient soil, continued with pure cultures of *Azotobacter* in sand with nutrients and finally carried out in liquid pure cultures of the same micro-organisms. When sufficient evidence had been obtained that boron is an essential micro-element for *Azotobacter*, a few boron-deficient soils were investigated in order to test the possibility of determining the boron content of soils microbiologically along this way.

The activity of the *Azotobacter* was measured by CO_2 production at 25°C as well as by the quantity of nitrogen fixed. All cultures were aerated with CO_2-free, sterile air.

Different carbon sources have been tried: mannite, glucose, calcium gluconate, sodium and calcium benzoates. The latter substance proved to be most useful in those cases where it was necessary to promote the growth of *Azotobacter* in competition with other soil microbes, many of which cannot use this substance as a carbon source. Moreover the pH remains fairly constant, which was not the case when sodium benzoate was used.

All chemicals had to be purified to the utmost; as glassware generally contains boron, and even quartz of quartz vessels, such as Vitrosil, is made better fusable with boron, it was finally decided to use aluminium kettles for the experiments in liquid cultures. Water was redistilled in an apparatus with an aluminium cooler.

METHODS

Purification of quartz sand and chemicals

Quartz sand: to one kg dry quartz sand, which had been repeatedly washed with distilled water, are added 5 ml acetic acid and 200–300 ml methyl alcohol in an aluminium pan. The mixture is heated on a waterbath and when the methyl alcohol has been evaporated, the same quantity is added anew. This is repeated 5 times. Finally the sand is dried at 105°C, also to get rid of residual acetic acid.

Saccharose, glucose and mannite: 50 ml of a saturated aqueous solution are put in a platinum dish and $\frac{1}{2}$ ml acetic acid and 50 ml methyl alcohol are added. When the methyl alcohol has been evaporated, the same quantity is added anew. This is repeated 5 times. The salts are dried at 105°C.

K_2SO_4, $MgSO_4$, $NaCl$, $FeCl_3$, $MnSO_4$, $ZnSO_4$,

$CuSO_4$ and Na_2MoO_4. Each salt is separately dissolved in boron-free water. To 10 ml of the saturated solution are added $\frac{1}{2}$ ml acetic acid and 50 ml methyl alcohol in a platinum dish on a waterbath; 5 times repeated; dried at 105°C. According to Kelsey and Dietrich[9]) the methyl borate ester has to be formed in the absence of water. Treadwell[13]), however, adds water during the esterification of boric acid with methyl alcohol. It is necessary to dissolve the different salts in water, prior to purification, otherwise the crystals might occlude notable quantities of boron which are not removed with methyl alcohol.

H_3PO_4. 10 g in 50 ml methyl alcohol. Heated in a platinum dish on waterbath. Same procedure as above.

Phosphates. In our first experiments K_2HPO_4 was used. As the purification of this substance proved to be difficult, KH_2PO_4 was used, which can be purified more easily, while the pH may be regulated by adding B-free CaO or $CaCO_3$. Finally it was decided to start from H_3PO_4 and neutralize this substance with the calculated amount of CaO or $CaCO_3$.

Benzoic acid. 10 g are dissolved in 50 ml methyl alcohol with $\frac{1}{2}$ ml acetic acid. Same procedure as above. Drying of benzoic acid at 105°C must be done more careful and as short as possible, otherwise the substance might be lost by sublimation. Ca-benzoate is made by adding the calculated quantity of CaO to the purified benzoic acid.

CaO. To 25 ml of a saturated Ca-acetate solution are added $\frac{1}{2}$ ml acetic acid and 50 ml methyl alcohol in a platinum dish. Same treatment as above. After drying at 105°C the substance is treated in an electric furnace at 1000°C and transformed into CaO.

Water. Glass distilled water is redistilled in an apparatus with an aluminium cooler, collected and stored in ordinary aluminium water kettles. The bacteria of one culture tube with *Azotobacter* are suspended in *ca* 10 ml B-free water. About $2\frac{1}{2}$ ml of this suspension to 500 g sand or soil is generally sufficient to bring the number of *Azotobacter* cells to appr. 1 million per gram of wet material.

CO_2 determination

500 g of soil or sterile sand are inoculated with a pure culture of *Azotobacter chroococcum* and put in metal cylinders varnished at

the inside with cellulose lacquer. Sterile, CO_2-free air is passed through the cylinder at a constant rate of 2 l per hour. The CO_2 is absorbed in spiral wash flasks containing 50 cc 1 *N* NaOH solution.

Total N determination

25 g soil or sand or 50 ml culture solution are destructed (Kjeldahl), transferred into a 1 l flask, neutralized with N-free NaOH and the ammonia distilled into a borate solution of pH 5.4, according to Ma and Zuazaga [10]).

Culture medium

Sand cultures. On 1 kg quartz sand (dry): 150 ml B-free water, 0.5 g K_2HPO_4, 0.2 0.5 g $MgSO_4$, 0.05 g. NaCl, 7.5 mg $FeCl_3$, 5 mg $MnSO_4$, 5 mg $ZnSO_4$, 1 mg $CuSO_4$, 1 mg Na-molybdate. Different carbon sources were tried: mannite 15–20 g/kg, Ca-gluconate 20 g kg and Ca-benzoate 2 g kg. In some cases 3 g $CaCO_3$ or 2 g Ca-acetate have been added to improve constancy of pH, which in general was kept between 6.5 and 7 at the beginning of the experiment and mounted to 7.5–8 towards the end.

Liquid cultures. To 1 l B-free water: 0.2 g KH_2PO_4, 0.2 g $MgSO_4$, 0.1 g NaCl, 0.1 g $CaSO_4$, 1 g $CaCO_3$, 15 mg $FeCl_3$, 10 mg $MnSO_4$, 10 mg $ZnSO_4$, 1 mg $CuSO_4$, 1 mg Na-molybdate. pH regulated at 6.7 by adding a few drops acetic acid.

EXPERIMENTAL

The first experiment was carried out with a sandy soil, on which sugarbeets showed severe symptoms of heart rot. In this case the chemicals were not yet purified. The soil to which 3 g/kg $CaCO_3$ had been added to regulate the pH, was not sterilised and was inoculated with *Azotobacter chroococcum*. As can be seen from the graph (Fig. 1) the CO_2 production increases by addition of 5 ppm B, reaching a maximum increase of 63% after 55 hours. Thereafter the distance between both curves deminishes gradually to 7% after 145 hours. This experiment seemed to indicate some influence of boron on CO_2 production, but was still far from convincing.

It was decided to carry on these experiments under more control-

led conditions and to use sterile quartz sand (instead of natural soil) inoculated with a pure culture of *Azotobacter chroococcum*.

The following experiment (Fig. 2) was carried out in duplicate using calcium gluconate as a carbon source. As this substance is finally transformed into $CaCO_3$ and CO_2, the pH changes are small. Though also in this case the chemicals had not been purified there is an increase of 69% in the average CO_2 production after 120 hours by the addition of 5 ppm B. The agreement between the duplicates is satisfactory.

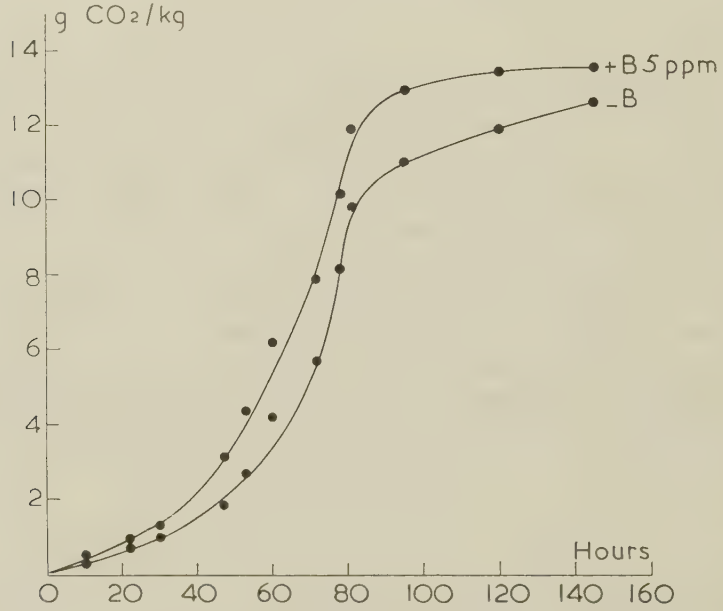

Fig. 1. The evolution of CO_2 by the microflora of a boron-deficient soil, with and without the addition of 5 ppm B.

As in both cases the cultures, which had received 5 ppm B produced less CO_2 after 24 hours than the controls, it might be possible that in the beginning this boron concentration was slightly toxic. At this point it seemed worthwhile to make a decisive attempt to show whether boron only stimulates the growth of *Azotobacter*, probably in an indirect way, or whether this element is an essential micro-nutrient for this organism. The chemicals and the sand were rigorously purified in the way described above. As a carbon source

mannite was used, to which 2 g Ca-acetate as a buffer substance, were added; the pH at the beginning was 7.0, the number of *Azotobacter* cells 3.7 . 10^5 g sand. Boron was added in increasing quantities of resp. 0, 0.5, 1.5, and 3 ppm.

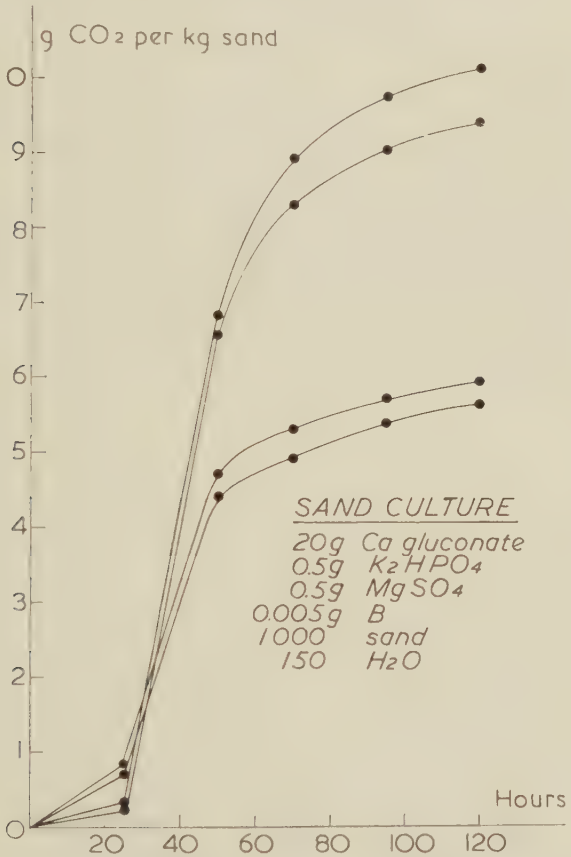

Fig. 2. The evolution of CO_2 by sand cultures of *Azotobacter chroococcum*, in duplicate. Lower curves without boron, upper curves 5 ppm B added.

The result of this experiment is shown in Fig. 3; it is evident that there is a fairly regular sequence of the curves in accordance with the quantity of boron added. As after 150 hours the carbon source of the sand which had received the highest amount of boron, seemed to be exhausted, 7.5 g mannite were added to this culture; the other cultures were mixed anew.

From this point *A*, however, the curves run nearly parallel; at the end of the experiment it could be shown that all the cultures were infected, most probably when the tubes had been opened. In the following table the results of the bacteria counts and the quantity of nitrogen fixed at the end of the experiment are summarised.

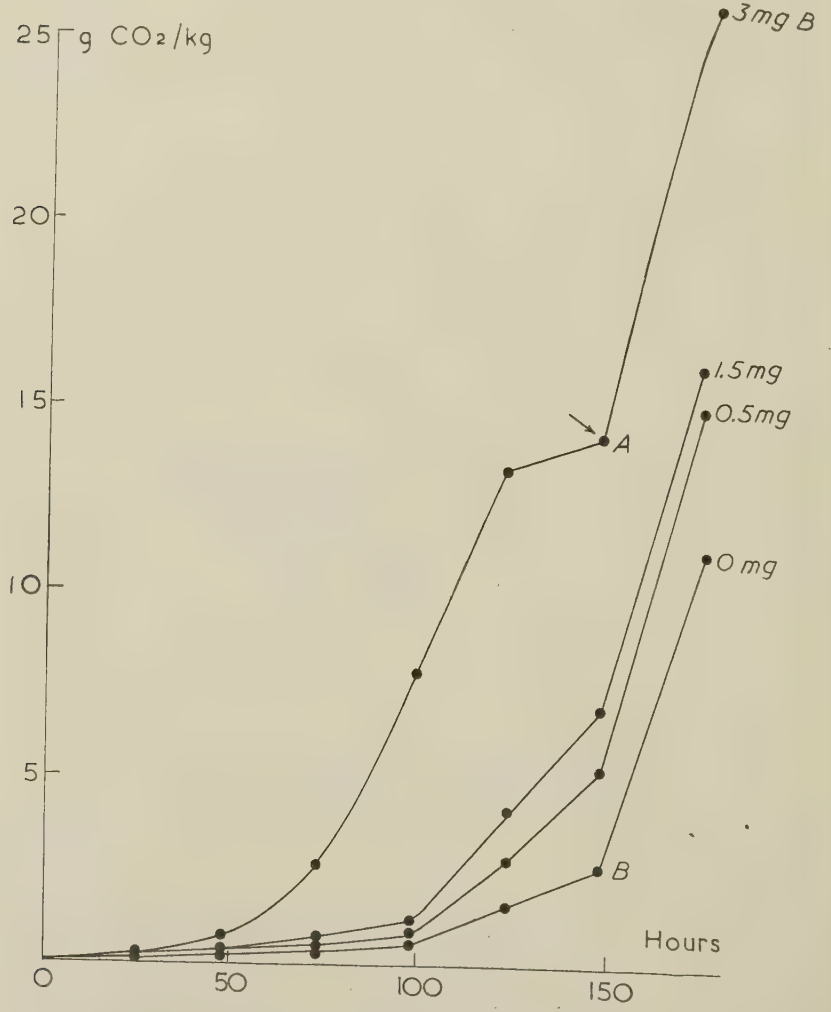

Fig. 3. The evolution of CO_2 by sand cultures (mannite) of *Azotobacter*, with increasing quantities of boron. Up to 150 hours the curves show a fairly regular sequence; thereafter the curves nearly run parallel, because the cultures had been infected.

TABLE I

Boron ppm	Number of *Azotobacter* cells per gram of soil	Total Nitrogen fixed mg in 500 g of sand
0	5×10^7	2.6
0.5	6.6×10^7	6.6
1.5	15×10^7	18.6
3.0	25×10^7	42.6

Number of bacteria at the beginning: 3.7×10^6 / g.
pH at the beginning: 7.0; pH at the end: 8.0.

The fact that by the addition of 3 ppm boron the quantity of nitrogen fixed has increased 17 fold, whereas the number of bacteria increased 5 fold, might be an indication, that boron plays a prominent part in the process of nitrogen fixation.

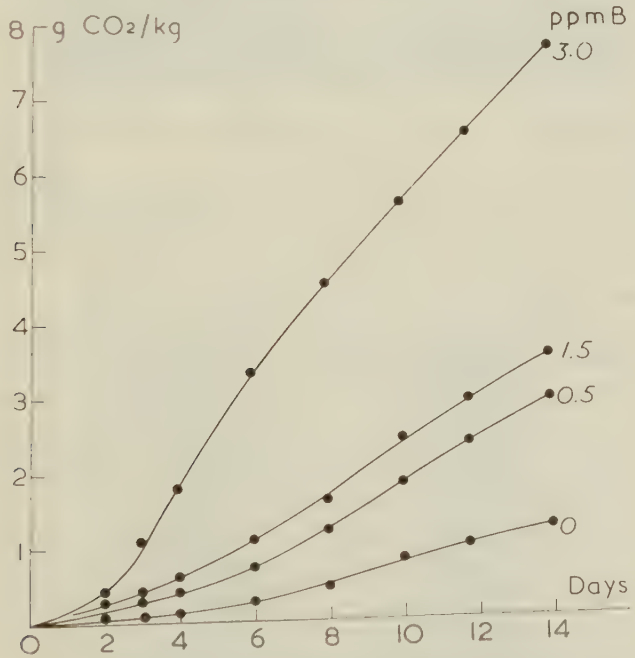

Fig. 4. The same experiment as that of Fig. 3. In this case the culture tubes were kept sterile.

The experiment was repeated, avoiding opening of the tubes. In this case 2 g CaO were added instead of Ca acetate, used in the former experiment. The results are plotted in Fig. 4. They fully

confirm the former experiment: the quantity of CO_2 produced with 3 ppm boron is 6 times as large as without boron, whereas 5 times as much nitrogen is fixed (see Table II).

TABLE II

Boron ppm	g CO_2 / kg sand in 14 days	mg N fixed / kg sand in 14 days
0	1.2	13.0
0.5	2.9	26.6
1.5	3.5	27.8
3.0	7.2	66.0

pH at beginning 8.3.
pH at end 8.3.

The sequence of the curves with 0, 1.5, and 3.0 ppm boron is quite regular, the position of the curve with 0.5 ppm boron, however, seems to be too high.

It is evident that boron not only stimulates the nitrogen fixation of *Azotobacter*, but that it is an *essential micro-element for this micro-organism.*

In the experiments with sterile quartz sand infected with a pure culture of *Azotobacter chroococcum*, it was observed that the sand had taken on a crump structure, in those cultures which had received an optimal quantity of boron (see Plate I).

The *Azotobacter* cells are surrounded by a gelatinous envelope, which cements the sand particles into more or less stable conglomerates. By the addition of boron the development of the *Azotobacter* had been promoted and it is curious to note that such a radical change in structure was brought about by the addition of 5 mg boron to one kilogram of sand.

It seemed worthwhile to repeat the experiments in liquid cultures. The first experiment was carried out in a quartz flask containing 475 ml mannite culture medium prepared with purified chemicals. During the experiment sterile CO_2-free air was passed through the medium; 0.4 ppm boron was added to one of the cultures.

CO_2 production	— B 8.4 g	+ B 7.9 g	in 9 days
mg N fixed	— B 59.1 ; 60.6	+ B 59.4; 60.6	

Although both cultures apparently were identical they differed in one aspect, viz. the colour of the bacteria. The culture which had

Plate I. The influence of the addition of boron on the structure of inoculated quartz sand.
Left the original sand. *Right* sand with culture solution, inoculated with *Azotobacter chroococcum*.
Middle the same after the addition of 5 mg boron to one kilogram of sand.

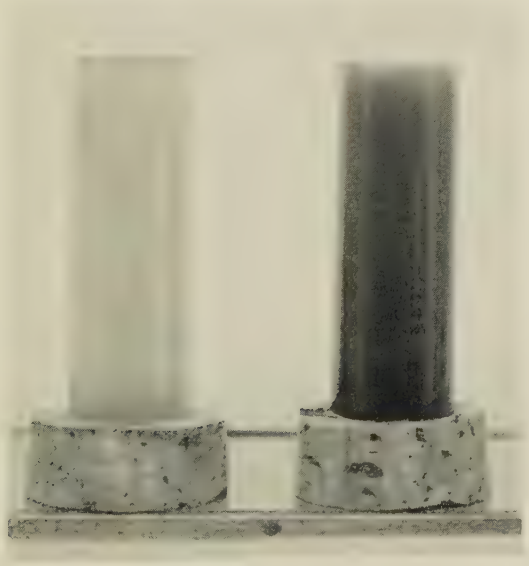

Plate II. Two cultures of *Azotobacter chroococcum*, left without boron (safe for traces in the culture medium), right with 0,4 ppm B added. The only difference is the colour, which is creamy yellow (—B) and dark-brown (+B). The number of *Azotobacter* cells and the quantity of N. fixed are exactly the same in both cases.

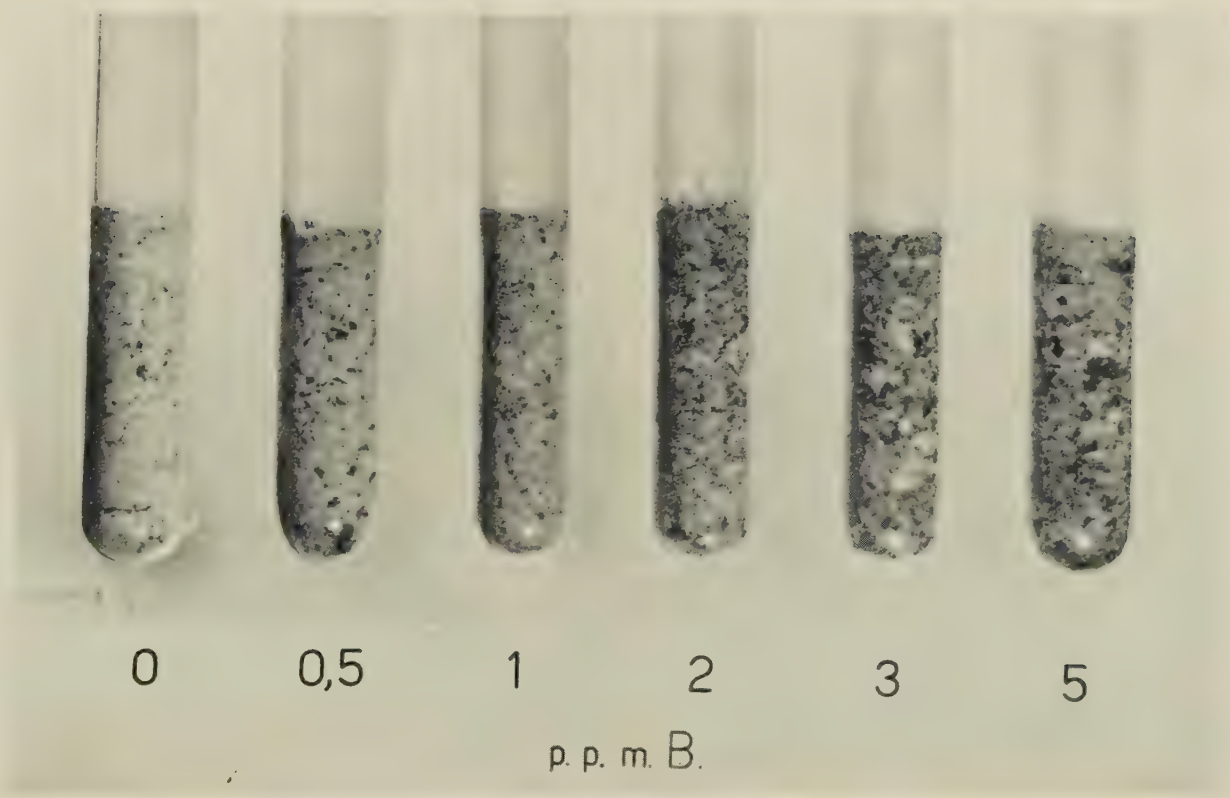

Plate III. Quartz sand cultures of *Azobacter chroococcum*, with Ca-benzoate as a carbon source and increasing quantities of boron. Note the increasing black colour of the sand, due to the greater number of *Azotobacter* cells and to the stimulation of pigment production.

not received boron, was creamy yellow, the one with 0.4 ppm boron was dark-brown as usual (see Plate II). This observation might indicate that the production of the dark-coloured substances in *Azotobacter* not only depends on the presence of minute quantities of copper (M u l d e r [11]), but also on the presence of boron. Even it seems that when only a limited quantity of boron is available, this colour-producing capacity is influenced first, whereas the nitrogen fixing-capacity still remains unaltered.

As even quartz vessels seemed to contain boron, the next experiment was carried out in aluminium water kettles, while the water

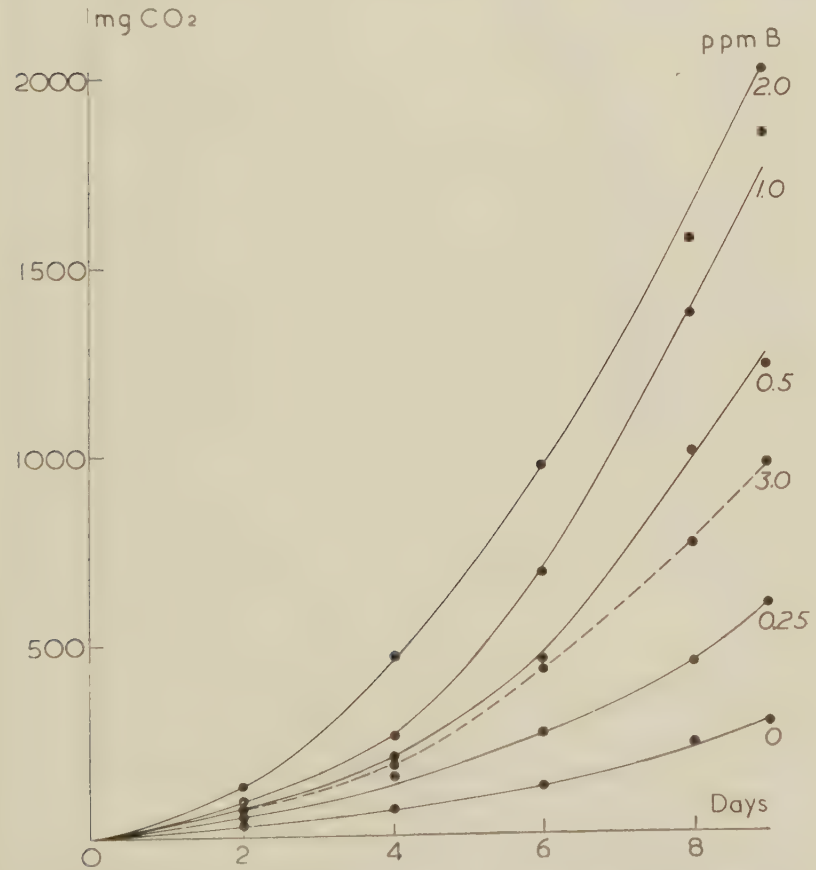

Fig. 5. CO_2 evolution of liquid cultures of *Azotobacter chroococcum* with increasing quantities of boron. The optimum is reached at 2 ppm, whereas 3 ppm has already a toxic effect.

was redistilled with an aluminium cooler. The results are summarized in Fig. 5 and 6.

There is a regular sequence of the quantity of CO_2 produced at different levels of boron, which runs parallel with the number of the bacteria and the quantity of nitrogen fixed. Original pH 6.7. Number of bacteria at the beginning 3.4×10^6.

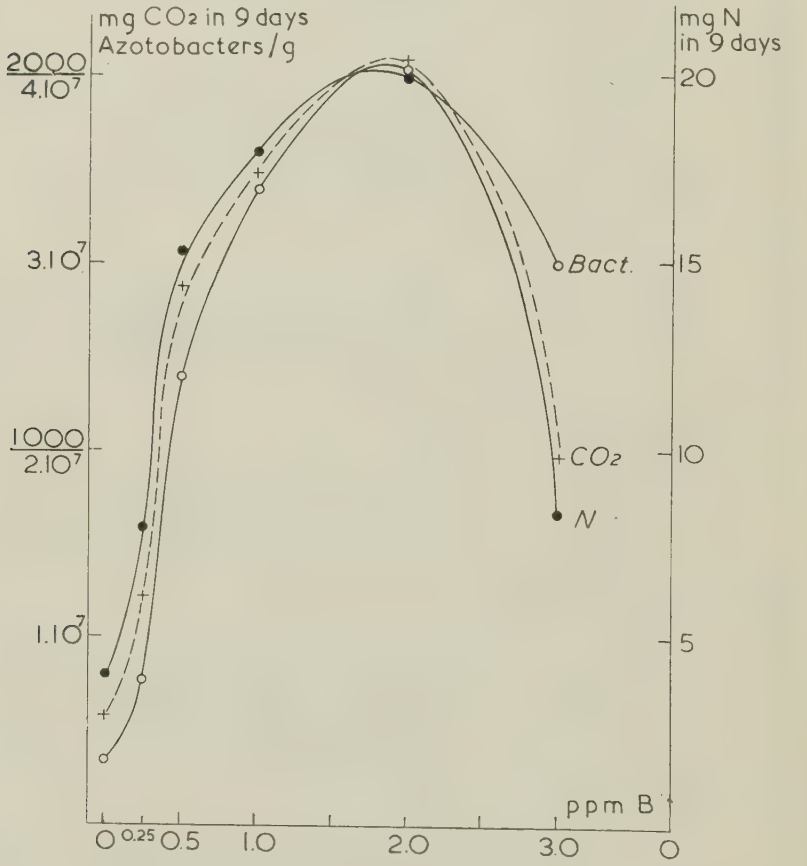

Fig. 6. The relation between the number of *Azotobacter* cells, the nitrogen fixed and the CO_2 produced in a series of liquid cultures with increasing quantities of boron.

It is evident that also in liquid cultures boron is indispensable and that it is an essential micro-element for *Azotobacter chroococcum*. The former observation that the influence of boron on nitrogen

TABLE III

ppm B	pH	CO_2 produced mg/500 ml	N fixed mg/500 ml	Number of bacteria, millions/ml
0	6.7	290	1.7	8
0.25	6.7	600	3.9	16
0.5	6.7	1230	12.0	32
1.0	6.7	1850	17.0	36
2.0	6.7	2020	20.0	40
3.0	6.6	970	8.4	30

fixation is much more pronounced than on multiplication has been confirmed in this experiment. As compared with the control, multiplication increased 5 fold at the optimal boron concentration of 2 ppm, whereas nitrogen fixation increased 12 times. As it seems a well established fact (P u r v i s [12]), B e r g e r [2])), that in plant metabolism boron is intimately related to protein formation this pronounced effect of boron on nitrogen fixation is not surprising.

In one aspect the liquid culture differs from the sand culture, viz. the optimal concentration of boron. In the former 2 ppm seems to give maximum development and at 3 ppm growth is decidedly reduced, whereas in the latter even 5 ppm boron does not have any inhibitory effect. These results have been fully confirmed in the next experiment where also an optimum is found at 2 ppm B (Table IV). These amounts are of the same magnitude as found by E d s o n *et al.* [3]) for *Penicillium chrysogenum* (3 ppm, which increased to 6 ppm by adding 10% $CaCO_3$ to the culture medium).

TABLE IV

ppm B	pH	CO_2 produced mg/500 ml/20 d	N fixed mg/500 ml/20 d	Number of *Azotobacter* cells millions/ml
0	6.8	145	2	25
0.5	6.9	565	6	50
1.0	7.1	805	9	80
2.0	7.5	**2000**	**20**	**140**
3.5	6.8	985	11	90
5.0	[illegible]	915	[illegible]	[illegible]

One might ask what is the reason that in sand cultures a much higher concentration of boron can be tolerated than in liquid cultures. As the concentration of boron in the sand cultures is calculated on the total mass of sand + culture solution and the

water content of the sand amounts to about 15%, the real concentration of boron in the culture solution as it is found between the sand particles amounts to 30 ppm, which is *ca* 15 times the optimal concentration in liquid cultures.

The sand cultures contain either $CaCO_3$ or organic Ca-salts, which finally are transformed into $CaCO_3$ with which boron might combine to form calcium borate. Though the solubility of the calcium borates is low, (2500 à 3000 ppm) it is so high, compared with the 30 ppm in the culture solution in the sand, that it seems hardly possible that this plays an important part. However P u r v i s [12]) says about this point: "Although it is generally agreed that liming produces boron deficiency in soil there is considerable difference of opinion as to the mechanism by which the deficiency is produced". That boron will be adsorbed at the surface of the sand particles does not seem very probable, as these particles consist of pure quartz.

To get an idea of the possibility and magnitude of the eventual adsorption of boron by the culture sand used in our experiments, the following preliminary experiment was made. 5 ppm boron as borax were added to 500 g quartz sand + 50 ml H_2O which was normally dressed with 0.5 g $Ca(H_2PO_4)_2$, 0.5 g K_2SO_4, 0.2 g $MgSO_4$, 0.1 g NaCl and 1 g Ca-benzoate (CaO added until pH 7.0). After being mixed carefully, the sand was allowed to stand for half an hour. After 450 ml H_2O were added, the sand was shaken for half an hour and filtered. Then B was determined in an aliquot part of the filtrate according to the method of T r e a d w e l l. It was shown that in this way only 51.6% of the boron added could be extracted from the sand; without entering into details of the way in which boron has been adsorbed, it is evident that this adsorption might easily account for the apparent higher concentration of boron, which *Azotobacter* can stand in sand cultures as compared with liquid cultures.

Though the proportionality of the growth of *Azotobacter chroococcum*, its nitrogen fixation and CO_2 production, with the boron content of the culture medium points to the possibility to determine boron microbiologically, it would be premature to use it to this end without further investigation. However it might be of interest to mention a few preliminary experiments in this direction.

Three soils of different origin and divergent boron contents (see

TABLE V

Soil	pH original	pH after addition of CaO	pH at end of exp.	Org. matter content %
Quartz sand. (Curve *S*).	—	6.8	7.1	0
B-deficient, cultivated sandy soil. (Curve *2*)	5.6	6.5	6.9	10.0
B-deficient sandy soil from a reclamation. (Curve *1*)	5.1	6.1	7.0	1.1
Light clay soil, 5% $CaCO_3$, well provided with boron. (Curve *3*). .	6.8	—	7.1	5.4

Table V) were compared with pure quartz sand. In all cases calcium benzoate was used as a carbon source (1 g Ca-benzoate on 500 g soil, other nutrients and micro-elements as indicated above). Calcium benzoate was chosen because *Azotobacter* can use this substance fairly well, whereas it is much less available to other micro-organisms. It seemed necessary to ascertain to what extent the proportionality between CO_2 production, N-fixation, multiplication of the *Azotobacter* cells and boron content of the sand would persist, when instead of glucose or mannite, Ca-benzoate was used as a carbon source. As might be seen from the graph in Fig. 7, the proportionality is retained, though the quantity of CO_2 produced is much smaller than when mannite or glucose were used. The effect of the addition of boron on the black colour produced by *Azotobacter* is very striking, as can be seen on Plate III *).

One of the difficulties encountered is that the pH of some of these soils was between 5 and 6, which is too low for the growth of *Azotobacter*. By the addition of CaO the pH was raised to a more suitable value between 6 and 7, which, however, might have influenced notably the availability of boron in the soil.

The soils were inoculated with an *Azotobacter* strain which had been isolated from the roots of oats on a culture medium which contained Na-benzoate.

*) After these investigations had been concluded, the interesting paper of Anderson and Jordan[1]) on pigmentation of *Azotobacter* as influenced by the interactions of boron and copper came to our attention. Optimum pigmentation occurred at concentrations of 70 ppm boron and 1 ppm copper. There is, however, a discrepancy between their results and ours; in their experiments *Azotobacter* can stand a concentration of not less than 112 ppm B, whereas in our experiments in liquid cultures the optimum is at 2 ppm (5 ppm in sand) and higher amounts are decidedly toxic.

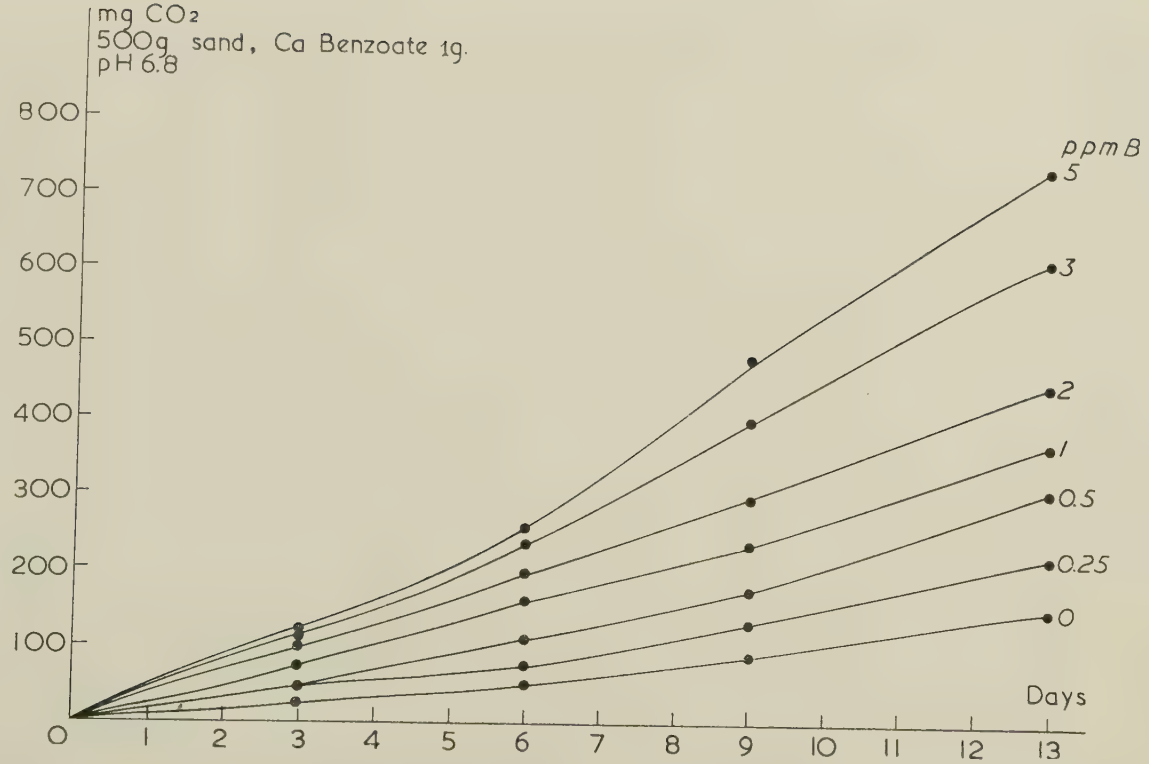

Fig. 7. CO_2 evolution in sand cultures with increasing quantities of boron inoculated with *Azotobacter* and calcium benzoate as a carbon source. The regular sequence of the curves is a "conditio sine qua non" for the microbiological determination of boron in soils.

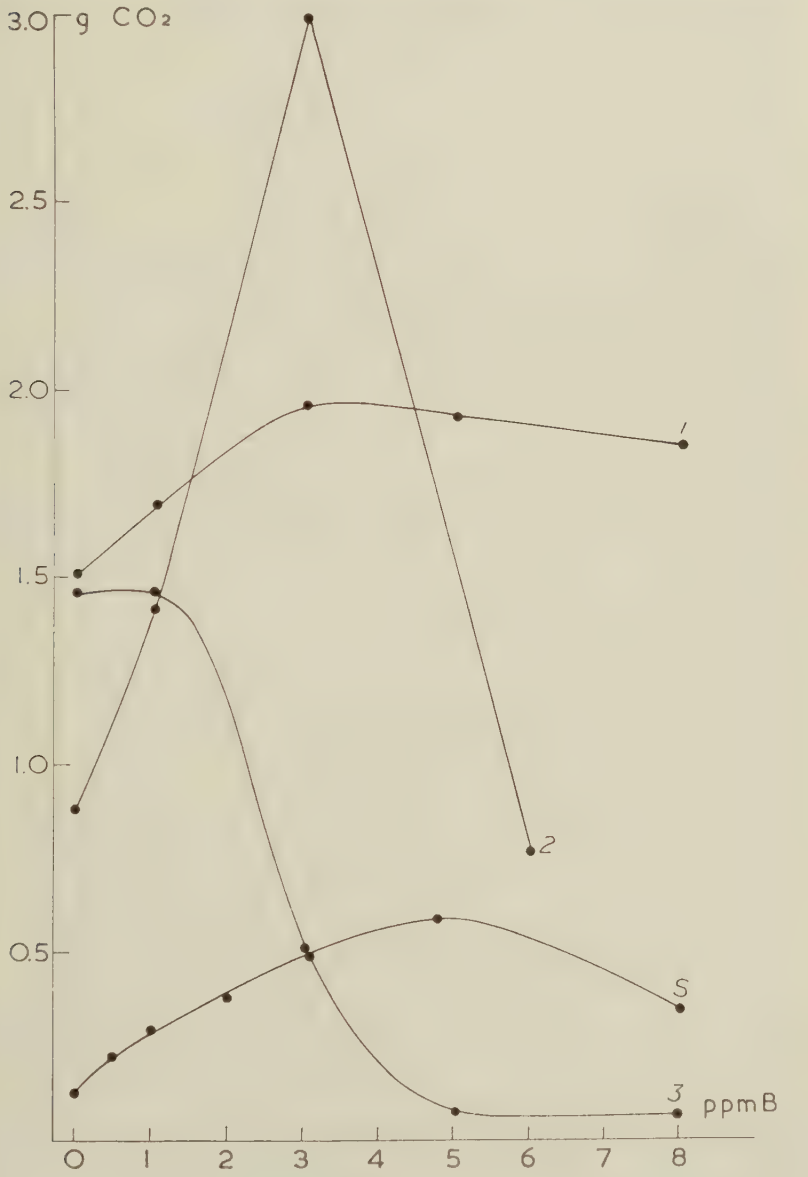

Fig. 8. CO_2 evolution of three different soils and a sand culture, 0.2% Ca-benzoate as a carbon source and inoculated with *Azotobacter*. The different types of curves are discussed in the text.

In Fig. 8 the total amounts of CO_2 produced by these soils in 11 days are plotted against the quantities of boron added. Three types of curves are to be distinguished:

A. Addition of boron causes a sharp increase in the microbial activity, up to a certain point followed by a rapid decrease (curve *2*).

B. Addition of boron does not increase CO_2 evolution and more boron causes a rapid decrease (curve *3*).

C. Addition of boron causes a moderate increase in CO_2 evolution; with more boron there is no perceptible or only a slight decrease of the microbial activity to be observed (curve *1*).

Type *A* refers to a soil, which is decidedly deficient in boron, but the question remains why in this case the maximum quantity of CO_2 produced is twice as much as in the case of curve *3* where the soil is well provided with boron. Attention is drawn to the fact that the former soil has the highest content of organic matter.

Type *B* is typical for a soil rich in boron. It contains so much boron that the addition of 2 ppm B has already a toxic effect.

Type *C*. The increase in CO_2 production in the beginning might be an indication that the soil is deficient in boron. The fact, however, that there is only a slight decrease when more B is added points to an adsorption of boron by the soil. Even at 8 ppm the CO_2 production still amounts to 95% of the optimum at 3 ppm, whereas in the case of curve *3* only 5% remains.

Also in these preliminary experiments there seemed to be some indication that the humus content of the soil in some way or another is related to the availability of boron and its influence on CO_2 production by *Azotobacter* in such a sence, that the greater the humus content, the more pronounced is the effect of boron on CO_2 production. Already in 1922 V o i c u [14]) reported a connection between the humus content of the culture medium and the sensitiveness of *Azotobacter* towards boron. Our experiments show that in soil the behaviour of boron is far more complicated than was anticipated. Microbiological investigation can be a helpful tool to unravel these different factors which play a part in these processes. The fact that *Azotobacter* wants a relatively large amount of boron for its growth, 2 5 ppm, whereas a normal sandy soil might contain *ca* 5–10 ppm B, makes it necessary to use rather large amounts of soil; liquid cultures, to which a small amount of soil is added as in the case of the *Aspergillus* method, are fully out of question. This

in combination with the complicated behaviour of boron in soils makes a microbiological determination of boron with the aid of *Azotobacter* less attractive for the time being.

SUMMARY

1. It was shown that boron is an essential micro-element for *Azotobacter chroococcum*.

2. Multiplication, CO_2 production, N fixation and pigmentation are closely related to the boron content of the culture medium. Of these, pigmentation is most susceptible to slight boron deficiency; with an easily assimilable carbon source (mannite, glucose) nitrogen fixation seems to be more susceptible than multiplication to boron deficiency.

3. With increasing gifts of boron, the CO_2 production curves in quartz sand with nutrients as well as in liquid cultures follow each other at regular sequences.

4. The optimum boron content for normal development of *Azotobacter* amounts to *ca* 2 ppm in liquid cultures, to 5 ppm in quartz sand cultures, whereas in soils sometimes 8 ppm B are tolerated, without noticeable loss of activity.

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REFERENCES

1. Anderson, G. R. and Jordan, J. V., Some factors affecting pigmentation of *Azotobacter chroococcum*. Agr. and Food Chem. **1**, 523–526 (1953).
2. Berger, K. C., Boron in soils and crops. Advances Agron. **1**, 321–351 (1949).
3. Edson, S. N., Novak, A. F. and Smith, F. F., A microbiological assay for available boron in soils. Soil Sci. Soc. Florida Proc. **X**, 94–99 (1950).
4. Eyster, C., Necessity of boron for *Nostoc muscorum*. Nature **170**, 755 (1952).
5. Hanna, W. J. and Purvis, E. R., Effect of borax and lime on activity of soil micro-organisms in Norfolk fine sandy loam. Soil Sci. **52**, 275–279 (1941).
6. Herzinger, F., Beiträge zum Wirkungskreislauf des Bors. Bodenk. u. Pflanzenern. **16**, 141–168 (1940).
7. Jogeswari, L., Trace elements nutrition of fungi. The effect of boron, zinc and manganese on *Fus.* spec. Proc. Indian Acad. Sci. **28**, 177–201 (1948).
8. Jordan, J. V. and Anderson, G. R., Effect of boron on nitrogen fixation by *Azotobacter*. Soil Sci. **69**, 311–319 (1950).
9. Kelsey, E. B. and Dietrich, H. G., Semi-micro qualitative analysis. The Macmillan Company, New York (1947).
10. Ma, T. S. and Zuazaga, G., Micro Kjeldahl determination of nitrogen. Ind. Eng. Chem. Anal. Ed. **14**, 280 (1942).
11. Mulder, E. G., Over de betekenis van koper voor de groei van planten en micro-organismen. Thesis, Wageningen (1938).
12. Purvis, E. R., The present status of boron in American agriculture. Soil Sci. Soc. Amer. Proc. **IV**, 316–321 (1939).
13. Treadwell, F. P., Kurzes Lehrbuch d. Anal. Chem. **II**, 364–365, Wien (1948).
14. Voicu, J., Influence de humus sur la sensibilité de *l'Azotobacter chroococcum* vis à vis de bore. Compt. Rend. **175**, 317–319 (1924).

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MOLYBDENUM IN RELATION TO GROWTH OF HIGHER PLANTS AND MICRO-ORGANISMS

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INTRODUCTION

In a previous paper [33]) the author has dealt with experiments on the importance of molybdenum for green plants and for micro-organisms. These experiments had been carried out with nutrient-solution cultures. No soils could be found on which the plants responded to added molybdenum. Shortly after the publication of the above-mentioned paper, however, it was found that molybdenum deficiency may occur rather frequently on certain soils in the Netherlands. The discovery of a molybdenum-deficient soil type in this country was more or less fortuitous. In 1949 the author carried out pot experiments with molybdenum-deficient Australian soils received from Southern Australia by courtesy of Prof. Prescott and Prof. Piper, The Waite Institute, Adelaide. Clovers growing on these soils responded clearly to added molybdenum, as might be expected from a number of experiments carried out by Australian workers [2]) [3]).

The soils were slightly acid and had a high content of ironstone. Since similar conditions may be found in the Netherlands in low moor peat soils in the neighbourhood of brooklets having their origin in heathy regions, pot experiments were carried out with soils of this type. Molybdenum deficiency appeared to be quite common in cauliflower and leguminous plants growing on these soils.

A more complicated case of molybdenum deficiency was found on

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very acid soils of different type (sandy, low moor, high moor). Cauliflower was the plant which responded particularly clearly to molybdenum dressings when growing on these soils. White clover, inoculated as described on p. 385, did not respond. Since available molybdenum, as determined with the *Aspergillus niger* technique, was found to be relatively high, it was obvious that interaction by some other soil constituent was responsible for the occurrence of molybdenum deficiency in cauliflower growing on these acid soils.

The present paper deals with the results of a number of pot experiments with mainly leguminous crops and cauliflower growing on molybdenum-deficient soils. In these experiments the influence of certain soil characters on effect of added molybdenum was studied. In some instances the pot experiments with green plants were paralleled by microbiological assay tests so that a comparison could be made of the uptake of molybdenum from various soils by *Aspergillus niger* and by higher plants. The results of a number of field experiments are also recorded.

EXPERIMENTAL METHODS

The pot experiments were carried out in glass jars which were cleaned first with hot water containing a detergent, and thereafter with hot 15 percent nitric acid. After removing the acid with tap water the containers were rinsed twice with glass-distilled water. In some cases enamelled Mitscherlich pots were employed.

The nutrient salts used for the pot and field experiments were tested for molybdenum contamination. Only those salts which were found to be practically free from molybdenum were utilised. In the case of nutrient-solution cultures of higher plants and of *Aspergillus niger* the method of purification described by Hewitt and Hallas [22]) was employed. This methode depends on the co-precipitation of molybdenum and copper sulphides.

For watering the plants glass-distilled water was mostly used.

In the experiments with leguminous plants the seed was always inoculated with an effective strain of *Rhizobium*. To ensure that the inoculation would be successful, the seed was mixed before sowing with a slightly acid molybdenum-deficient peat soil which had been inoculated with a dense suspension of *Rhizobium* cells.

In the case of field experiments the crops were grown on small plots separated by uncultivated strips to prevent contamination of the control plots.

Analytical methods. Molybdenum was determined chemically according to the thiocyanide method as modified by Evans *et*

al. [15]) and in some instances according to the dithiol method of Piper and Beckwith [42]). The biological method with *Aspergillus niger* was also used [34]). For the estimation of available molybdenum in soil the latter method was employed. The pH of the nutrient solution used in this test was brought to a value of 2.0 [38]). Manganese was determined according to the periodate method and the formaldoxime method [26]), nitrite according to the Griess–Romijn method [44]).

POT AND FIELD EXPERIMENTS WITH SOILS RICH IN IRONSTONE

Pot experiments with leguminous plants

In preliminary pot experiments, white clover, subterranean clover and peas were grown on two Australian soils, dressed as follows: K_2HPO_4 0.6 g, $MgSO_4.7H_2O$ 0.24 g, $MnSO_4.4H_2O$ 5 mg, $CuSO_4.5H_2O$ 5 mg, $ZnSO_4.7H_2O$ 2.5 mg per pot, containing approximately two kg of soil. Some pots received 5 mg of $Na_2MoO_4.2H_2O$ per pot.

A pronounced response to added molybdenum was found in the clovers but not in the pea plants (Table I). In the absence of added molybdenum the clovers had a yellow-green colour due to nitrogen deficiency. Dressed with 5 mg of Na_2MoO_4 they had dark green leaves.

TABLE I

Effect of molybdenum on nitrogen fixation and yield of white and subterranean clover grown on Australian soils

Soil from	pH of soil	$Na_2MoO_4.2H_2O$, mg per pot	White clover		Subterranean clover	
			Dry weight, g per pot *)	Nitrogen in plants, mg per pot *)	Dry weight, g per pot *)	Nitrogen in plants mg per pot *)
Tasmania. . .	6.2	0	4.2	110.5	12.9	272.0
,, . . .	6.2	5	12.2	382.0	16.8	428.5
S. Australia. .	5.2	0	4.3	93.4	11.1	243.0
,, . .	5.2	5	5.9	170.0	17.3	395.0

*) Averages of duplicate values.

Subsequently a number of pot experiments were carried out with soils resembling those from Australia. Such soils may be found in the Netherlands along brooklets having their origin in acid heathy regions. They are rich in ironstone and have often a high content of organic matter. Table II gives the results of an experiment in which increasing amounts of sodium molybdate were added to a

TABLE II

Effect of increasing amounts of molybdenum on nitrogen fixation and yield of white clover grown on an acid low moor peat soil rich in ironstone

$Na_2MoO_4.2H_2O$, μg per pot *)	Dry weight, g per pot **)	Nitrogen in clover		Number of nodules **) per glass-jar bottom †)	Molybdenum in clover, ppm **)
		% of dry matter **)	mg per pot **)		
0	2.1	2.46	51.6	456	< 1.0
10	2.7	2.63	71.7	368	1.8
50	4.1	3.20	131.0	254	2.4
100	4.7	3.24	152.0	151	3.7
500	4.8	3.32	158.7	87	4.3
1000	4.8	3.22	155.0	116	3.3
2500	4.7	3.19	150.0	123	6.5
5000	4.6	3.24	147.9	116	13.7
10000	4.8	3.13	151.0	107	25.4
20000	3.8	3.34	127.0	109	80.0
50000	4.4	3.32	147.0	70	183.1

*) Each pot contained about 500 g of soil.

**) Averages of duplicate values.

†) Only those nodules were counted which could be seen through the glass-jar bottom.

soil of pH 5.8 containing 60% of organic matter. The experiment was carried out in glass jars containing approximately 0.5 kg of soil. The basic dressing consisted of 0.6 g of K_2HPO_4 per pot. It will be seen that the white clover gave a pronounced response to the added molybdenum. In the absence of added molybdenum the plants had a yellow-green colour due to nitrogen deficiency. In this case the number of nodules was considerably higher than when molybdenum had been added in adequate amounts.

The molybdenum-deficient nodules were smaller than those of normal plants while their colour was not pinkish as usual but yellow or brown-gray. This is in agreement with the pea experiments in nutrient solutions (cf [33]).

The results of Table II show that with increasing amounts of added molybdate the molybdenum content of the white clover rose readily. A dressing of 20 mg per $\frac{1}{2}$ kg of soil, which is comparable with 40 kg per ha, gave a value of 80 ppm, a concentration which is usually considered as highly toxic to cattle ([6]) [11]) [17]).

In a second pot experiment with the same soil, glass jars of two liter capacity were used each containing approximately two kg of soil. White and red clover were the test plants. Both clovers

gave a pronounced response to the added molybdenum as will be seen from Table III. The control plants gave higher yields than in the preceding experiments presumably due to the fact that more soil nitrogen was available.

TABLE III

Effect of added molybdenum on nitrogen fixation and yield of white and red clover

$Na_2MoO_4 . 2H_2O$, mg per pot	White clover			Red clover		
	Dry weight, g per pot *)	Nitrogen in clovers		Dry weight, g per pot *)	Nitrogen in clovers	
		% of dry matter *)	mg per pot *)		% of dry matter *)	mg per pot *)
0	4.19	2.85	119	4.89	3.06	150
10	5.78	3.76	217	6.87	3.66	251

*) Averages of duplicate values.

Pea and bean (*Phaseolus*) plants grown in the same way as the clovers did not respond to the added molybdenum. Both plant species were able to fix adequate amounts of atmospheric nitrogen in the absence of added molybdenum.

In a subsequent experiment white clover was grown in 33 different soils mostly of the above-mentioned type. Neubauer jars containing approximately 500 g of soil were used in this experiment. The basic dressing consisted of 0.5 g of K_2HPO_4 and 50 mg of $MgSO_4 . 7H_2O$. Some of the pots also received 0.5 mg of $Na_2MoO_4 . 2H_2O$. In order to eliminate a possible sodium effect of the molybdate, 0.6 mg of $Na_2CO_3 . 10\,H_2O$ was added to all pots. All soils were inoculated with a suspension of *Rhizobium*.

The results of this experiment are given in Table IV (see also Plate I). It will be seen that only in 5 out of 33 soils no increase in nitrogen fixation and yield of white clover was obtained. In all of the other cases molybdenum gave a material rise in nitrogen fixation and yield of dry matter of the white clover. In general the molybdenum-deficient plants had considerably more nodules than those provided with molybdenum. The nodules were smaller, however, and had a yellow colour as contrasted to the pink colour of normal nodules. As a result of the poor nitrogen nutrition the molybdenum-deficient plants had yellow-green leaves.

That the beneficial effect of molybdenum in this experiment depended mainly on improved nitrogen fixation may also be con-

Plate I. Effect of molybdenum on growth of white clover. *Left*: without added molybdenum; *right*: supplied with 0.5 mg $Na_2MoO_4.2H_2O$ per pot. Soils: 2 (above), 5, 12, 15, 16 and 20 (cf Table 4).

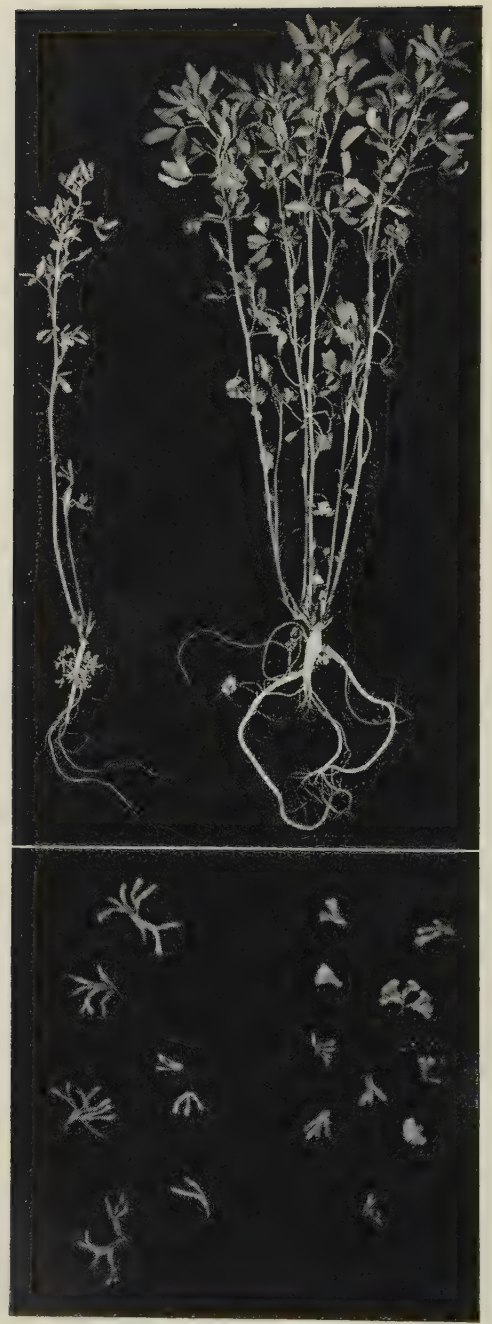

Plate II. Effect of molybdenum on growth of lucerne (above) and development of nodules (below). *Left*: without added molybdenum; *right*: dressed with 4 kg $Na_2MoO_4.2H_2O$ per ha.

cluded from the results of a subsequent experiment with white clover in which a number of pots were provided with combined

TABLE IV

Effect of molybdenum on nitrogen fixation and growth of white clover grown on sandy and peaty soils

Soil			No molybdenum				0.5 mg $Na_2MoO_4.2H_2O$ per pot			
No	Type	pH	Dry matter†), g per pot	Total N †)		Mn †), ppm ††)	Dry matter†), g per pot	Total N †)		Mn †), ppm ††)
				% ††)	mg			% ††)	mg	
1	sandy, Fe *)	5.9	1.74	2.64	45.9	—	1.96	3.19	62.5	—
2	,,	5.3	0.74	2.42	18.0	—	1.94	3.02	58.3	—
3	,, ,,	5.6	1.47	3.47	50.8	—	2.28	3.37	76.9	—
4	,, ,,	5.4	2.71	2.93	79.3	72	3.81	3.19	121.6	62
5	,, ,,	5.8	1.99	3.17	63.0	67	3.70	3.42	126.4	69
6	peat, Fe	5.7	2.52	3.39	85.3	77	3.17	3.22	102.3	78
7	,, ,,	5.0	3.12	3.23	101.0	87	2.88	3.20	91.9	84
8	,, ,,	5.1	2.46	3.23	79.6	176	3.40	3.36	114.2	215
9	,, ,,	5.2	2.74	3.24	88.8	122	3.73	3.28	122.0	137
10	,, ,,	5.3	3.50	3.63	126.7	182	2.96	3.24	95.9	203
11	,, ,,	5.7	2.87	3.25	93.0	74	3.70	3.24	120.0	74
12	,, ,,	6.0	0.28	2.42	6.6	—	1.26	3.22	40.4	—
13	,, ,,	5.9	1.64	3.09	50.9	49	2.35	3.19	74.8	47
14	sandy, Fe	6.1	1.47	3.02	44.5	90	2.89	3.20	92.4	84
15	,, ,,	5.5	1.15	2.54	29.0	84	3.43	3.33	114.0	69
16	,, ,,	5.7	1.54	2.61	40.0	87	3.65	3.02	109.9	74
17	,, ,,	5.6	0.28	2.91	8.2	—	0.89	3.52	31.1	—
18	,, ,,	6.1	2.06	2.97	61.1	36	2.52	3.03	76.2	35
19	,, ,,	4.6	0.52	2.51	13.0	—	1.86	3.15	58.4	—
20	,, ,,	5.4	0.68	2.16	14.5	94	2.34	2.95	68.8	88
21	,, ,,	5.3	2.00	2.95	59.0	91	3.11	3.12	96.9	132
22	peaty, Fe	5.5	2.24	1.96	43.9	74	3.40	3.28	111.5	79
23	peaty **)	6.2	2.06	2.93	60.5	53	3.01	3.29	98.8	52
24	,, **)	5.2	2.69	3.50	93.9	295	3.93	3.36	132.0	309
25	,,	5.4	2.20	3.49	76.2	204	2.65	3.65	96.0	168
26	,, **)	5.2	2.83	3.48	98.1	290	2.74	3.57	97.6	204
27	sandy ***)	5.4	2.27	3.32	75.2	122	2.23	3.39	76.1	142
28	,, ***)	5.1	1.33	3.57	47.5	65	1.98	3.61	71.1	68
29	peaty, Fe	4.9	2.38	3.58	84.9	184	2.83	3.48	98.4	155
30	,, ,,	5.7	1.21	2.56	30.8	114	1.70	3.72	63.2	110
31	,, ,,	6.1	0.57	3.16	18.0	—	0.42	3.03	12.7	—
32	,, ,,	6.2	2.07	3.34	69.1	77	2.92	3.21	93.3	84
33	clay	6.2	1.33	3.17	42.1	114	2.36	3.26	77.0	117

*) Fe ironstone-containing soil.
**) Reclaimed high-moor peat.
***) Reclaimed heathy soils.
†) Averages of duplicate values.
††) On dry matter basis.

nitrogen in the form of KNO_3. Care was taken that no molybdenum was added as an impurity in the nitrogen compounds.

As will be seen from Table V a much more pronounced response to molybdenum was obtained in the absence of added combined nitrogen than in the presence of nitrate. Apparently the concentration of molybdenum in the clover plants, although inadequate for fixation of atmospheric nitrogen by the nodules, was high enough for nitrate assimilation and for other molybdenum-requiring reactions in the plants.

TABLE V

Effect of combined nitrogen and molybdenum on yield (g dry weight per pot)*) of white clover grown on molybdenum-deficient soils

Soil	No molybdenum		1 mg $Na_2MoO_4.2H_2O$ per pot	
	No combined N	Nitrate	No combined N	Nitrate
1297– 1	3.44	4.25	4.86	4.89
2	1.92	2.71	3.50	3.28
5	2.55	3.34	2.94	3.48
12	2.09	3.16	3.13	3.29
15	2.35	2.96	3.85	3.86
20	2.89	3.88	4.36	4.64
69	2.50	2.64	3.02	2.87

*) Averages of duplicate values.

Field trials with leguminous crops

On a number of soils which had responded clearly to molybdenum dressings in the pot experiments, field trials with white and red clover and with lucerne have been carried out. In most cases a clear response to the added molybdenum was obtained (Table VI, Plate II). As in the pot experiments the molybdenum-deficient plants showed a characteristic nitrogen deficiency. The number of nodules on these plants was consistently higher than that on plants dressed with molybdenum. On lucerne the nodules of the molybdenum-deficient plants were slender (Plate II). They had a yellow colour as contrasted to the pinkish colour of the compact molybdenum-supplied nodules.

In some instances the clovers and the lucerne grew almost normally on the control plots notwithstanding practically no nitrogen fixation had taken place without molybdenum dressings in the pot experiments in which the same soil was employed. This discrepancy may have been due to molybdenum uptake from the ,subsoil in the case of leguminous crops. Cauliflower, white cabbage

TABLE VI

Effect of molybdenum on yield *) and nitrogen content *) of white and red clover, lucerne and grass **)

Experiment	$Na_2MoO_4 . 2H_2O$ kg per ha †)	White clover		Red clover		Lucerne		Grass,
		airdry matter, q ††) per ha	N, % of dry matter	airdry matter, q per ha	N, % of dry matter	airdry matter, q per ha	N, % of dry matter	airdry matter, q per ha
1297–3 ***)								
1st year								
1st cutting	0	28.0	3.89	28.9	3.66	22.7	3.00	27.0
	2	34.8	3.66	37.4	3.49	36.2	3.77	25.6
	4	38.0	4.23	41.3	3.59	31.5	3.72	32.6
2nd cutting	0	21.3	3.73	26.7	3.06	15.9	2.67	—
	2	22.8	3.65	29.8	3.24	20.8	3.28	—
	4	25.0	4.06	23.9	3.14	21.0	3.35	—
2nd year								
1st cutting	0	37.1	3.61	69.4	2.93	26.8	2.26	-
	2	37.6	3.61	61.3	2.99	38.1	2.96	—
	4	42.1	4.18	68.6	3.10	35.7	2.27	—
2nd cutting	0	21.7	3.42	63.7	2.69	38.6	2.74	—
	2	23.2	3.42	51.7	2.91	58.1	2.81	—
	4	23.2	3.72	56.0	2.73	49.3	2.56	—
1297–4								
1st year								
1st cutting	0	17.9	2.99	9.3	2.56	6.5	2.23	43.5
	2	34.2	3.15	27.0	2.75	17.7	2.73	45.8
	4	30.4	3.01	26.5	2.83	26.3	3.47	37.1
2nd cutting	0	26.0	3.43	33.7	2.53	6.0	2.53	—
	2	33.5	3.54	38.7	3.13	23.2	3.60	—
	4	33.2	3.37	38.6	2.96	20.6	2.84	—
2nd year								
1st cutting	0	46.5	3.49	74.4	2.46	10.8	2.31	—
	2	37.7	3.94	55.9	2.82	19.1	2.94	—
	4	50.4	4.25	67.7	2.62	20.6	3.14	—
2nd cutting	0	22.3	—	—	—	30.8	—	—
	2	24.7	—	—	—	34.6	—	—
	4	27.9	—	—	—	39.5	—	—
1297–8								
1st year								
1st cutting	0	35.5	4.03	27.6	—	25.3	3.24	18.8
	2	37.6	3.95	38.1	—	38.2	3.48	23.7
	4	36.4	4.16	33.6	—	34.5	3.92	23.1
2nd cutting	0	22.9	4.25	24.3	3.79	23.6	3.81	—
	2	26.7	4.30	25.9	3.58	25.6	3.88	—
	4	27.9	3.70	28.2	3.68	27.2	3.57	—

*) Averages of duplicate values.

**) Mixture of *Dactylis glomerata*, *Lolium perenne*, *Lolium italicum* and *Phleum pratense*, dressed with calcium nitrate.

***) These experiments had been laid out on slightly acid ironstone-containing soils (cf Table VII). As a basic dressing dicalcium phosphate, potassium sulphate and magnesium sulphate were applied.

†) Molybdate was applied at the beginning of the experiment only.

††) 1 q = 100 kg.

potato, sugar beet and cereals gave a pronounced response to molybdenum when growing one of these soils (see p. 377–82).

When the leguminous plants grew older (second cutting of the year of sowing and subsequent year) the molybdenum-deficiency symptoms in general became less clear. Apparently the plants had succeeded in accumulating adequate amounts of molybdenum. This was particularly true of red clover as contrasted to lucerne which responded also in the second year. This difference between red clover and lucerne was shown clearly in Experiment 1297–4. In the first cutting the yield of red clover was increased nearly 200 percent by a molybdenum dressing; in the second cutting the increment was only 15 percent, whilst in the second year no effect at all was observed. Lucerne gave an increase of 300 percent in the first cutting, 250 percent in the second, 100 in the third and 30 in the fourth. Generally lucerne was affected more severely by molybdenum deficiency than the clovers. In a number of cases in which clovers grew normally in the absence of added molybdate, the yield of lucerne was increased considerably by a molybdenum dressing. The latter observation is in agreement with the finding of J e n s e n [24]) that nodules of lucerne require a higher concentration of molybdenum to function normally than those of subterranean clover.

Response of non-leguminous plants to molybdenum dressings

The liability of cauliflower and cabbage to molybdenum deficiency is a well-known fact ([12]) [21]) [31]) [43])).

Therefore c a u l i f l o w e r was grown on a number of the soils used in the above-mentioned pot experiments with white clover. The response of cauliflower plants to added molybdenum appeared to be very pronounced. In many cases the seed leaves were the only leaves of the control plants which showed a normal green colour. The second pair of leaves had a chlorotic appearance and did not attain a normal development (Plate IIIA). They were severely cupped and large parts of the laminae, beginning at the margins, died at an early stage. Subsequent leaves showed similar symptoms. Frequently the plants died at an early stage. Sometimes the molybdenum-deficient plants continued to grow. Such plants in a later stage developed the typical leaves known in the literature as whiptail (Plate IIIB).

Plate III. Cauliflower plants grown on a molybdenum-deficient soil. A: young plants; B: typical symptoms of "whiptail"; C: dressed with 4 kg $Na_2MoO_4.2H_2O$ per ha.

Plate IV. White cabbage grown on a molybdenum-deficient soil. A: severely deficient; B. moderately deficient; C: normal plants (dressed with 1 kg $Na_2MoO_4.2H_2O$ per ha)

TABLE VII

Effect of molybdenum on heart formation in cauliflower

Exp. field and soil	pH and org. matter, %	$Na_2MoO_4.2H_2O$ kg per ha	N-fertiliser **)	Classification of cauliflower (% of number according to diameter of heart) †)			
				no heart	1–8 cm	9–14 cm	>14 cm
1297–1, peaty *)	5.8, 40	0	cn	85	11	5	0
		0.5	,,	47	24	29	0
		1	,,	24	23	41	12
		2	,,	11	6	67	16
		4	,,	0	22	39	39
1297–2, peaty *)	5.8, 58	0	cn	96	4	0	0
		1	,,	33	67	0	0
		2	,,	0	88	12	0
		4	,,	25	50	25	0
1297–3, sandy	5.7, 16	0	cn	50	42	8	0
		1	,,	13	37	50	0
		2	,,	0	38	62	0
		4	,,	17	33	50	0
1297–4, sandy	5.6, 11	0	cn	94	4	2	0
		0	an	84	17	0	0
		0.5	cn	11	50	39	0
		1	,,	6	35	53	6
		2	,,	3	60	37	0
		2	an	0	50	44	6
		4	cn	0	61	33	6
1297–7, sandy	5.5, 11	0	cn	64	36	0	0
		1	,,	38	62	0	0
		2	,,	25	75	0	0
		4	,,	17	83	0	0
1297–8, peaty *)	5.9, 49	0	cn	50	45	5	0
		1	an	88	13	0	0
		2	cn	13	87	0	0
		2	an	11	72	17	0
		4	cn	0	71	29	0
1297–9, sandy	5.8, 10	0	cn	100	0	0	0
		1	,,	33	67	0	0
		2	,,	11	78	11	0
		4	,,	11	67	11	11

*) Low moor peat.

**) cn = calcium nitrate, an = ammonium nitrate.

†) Single values, control in triplicate.

The symptoms of molybdenum deficiency in cauliflower were also frequently observed on experimental fields. Table VII gives the results of 8 field trials. In these experiments the cauliflowers were harvested when the hearts had reached their maximal development. In most cases heart formation was absent in the plants not dressed with molybdenum. When provided with $Na_2MoO_4.2H_2O$ at the rate of $\frac{1}{2}$ kg per ha the development of the plants was considerably improved but this amount was inadequate for the production of normal cauliflowers. In most cases the best results were obtained at a dressing of 4 kg $Na_2MoO_4.2H_2O$ per ha.

C a b b a g e plants responded to added molybdenum similarly to cauliflower. Early death occurred less frequently, however, so that on the control plots many plants with poor hearts were found (see Plate IV). Cabbage required smaller amounts of molybdenum than cauliflower so that 1 kg of sodium molybdate, applied in 1951, gave nearly optimal yields in 1953 (Table VIII). Small differences in available molybdenum, as brought about by a different crop in the preceding year, affected a relatively large difference in appearance of the plants.

Other cruciferous plants which responded more or less clearly when grown on molybdenum-deficient soil were r a p e, t u r n i p and r a d i s h. In the absence of added molybdenum the first two plant species had mottled leaves as contrasted to the dark green leaves of the plants dressed with molybdate. This was the case in pot and field experiments. Rape gave higher yields upon application of molybdenum when grown on a number of soils in

TABLE VIII

Effect of molybdenum on heart formation in cabbage *) (Exp. 1297–1, 1953)

$Na_2MoO_4.2H_2O$, kg per ha	Classification of cabbage (% of number) according to diameter of heart			
	no heart	1–15 cm	16–20 cm	> 20 cm
0	66	13	16	5
1 (1951)	10	23	60	7
1 (1951 and '53)	10	3	70	17
2 (1951)	11	24	37	28
2 (1951 and '53)	3	17	67	13
4 (1951)	10	23	57	10
4 (1951 and '53)	10	17	56	17

*) Manuring: 160 kg N per ha as $Ca(NO_3)_2$, 300 kg K_2O per ha as K_2SO_4, 100 kg P_2O_5 per ha as $CaHPO_4$ and 20 kg MgO per ha as $MgSO_4.7H_2O$. Single values, control in duplicate.

glass jars but did not respond in field experiments. In the case of turnips molybdenum gave higher yields in a field experiment (Table IX). Molybdenum-deficient radish plants were chlorotic. Cupping of the leaves and necrosis of the leaf margins occurred frequently (see Plate VA). The yield was considerably reduced.

TABLE IX

Effect of molybdenum on yield of turnips *) (Exp. 1297–9, 1953)	
$Na_2MoO_4.2H_2O$, kg per ha	Turnips (roots), q **) per ha
0	29
1	47
2	36
4	37

*) Manuring: 80 kg N per ha as $Ca(NO_3)_2$, 50 kg P_2O_5 per ha as $CaHPO_4$, 100 kg K_2O per ha as K_2SO_4 and 10 kg MgO per ha as $MgSO_4.7H_2O$. Single values, control in triplicate.

**) 1 q = 100 kg.

Lettuce responded very clearly to added molybdenum when growing on soil of the above-mentioned type. In the absence of added molybdenum its leaf colour was pale yellow-green and necrotic spots occurred on the lower leaves. These symptoms are in agreement with those obtained by Warington [48]) in nutrient-solution experiments. Its growth was very poor so that the yields were low (see Table X).

TABLE X

Effect of molybdenum on yield of lettuce *)		
$Na_2MoO_4.2H_2O$, kg per ha	Yield, fresh weight, q **) per ha	
	1297–3	1297–9
0	64	39
1	—	121
2	101	149
4	110	247

*) This crop was sown in the autumn and had to be harvested at a young stage. Manuring similar to that recorded in Table IX. Single values, control in triplicate.

**) 1 q = 100 kg.

Spinach gave also a very pronounced response to added molybdenum (Table XI). The control plants had pale yellow

leaves as contrasted to the dark green leaves of plants supplied with a small amount of molybdate. Many leaves showed cupping and necrosis of the leaf margins (Plate VB). Since the growth of this plant is quick, it may be used as an indicator plant for molybdenum deficiency. Johnson *et al.* (25) who determined the response to added molybdenum of a number of crop plants, grown on a molybdenum-deficient soil, placed spinach in the highest response group.

TABLE XI

Effect of molybdenum on yield, fresh weight q per ha, of spinach

$Na_2MoO_4.2H_2O$ kg per ha	Experiment				
	1297–1	1297–3 *)	1297–4	1297–7 *)	1297–11
0	44 ± 3	274 ± 36	112 **)	284 ± 14	131 ± 13
0.5	79 ± 20	400 ± 82	—	338 ± 16	161 ± 23
1	102 ± 13	397 ± 76	—	374 ± 44	191 ± 36
2	—	—	243 **)	—	—
4	126 ± 5	297 ± 31	—	385 ± 51	162 ± 24

*) Harvested at a late stage.
**) Single values.

Sugar beet also required a molybdenum dressing when growing on ironstone-containing soil. Molybdenum-deficient sugar beet had highly chlorotic leaves which showed a pronounced cupping similar to molybdenum-deficient cauliflower leaves (cf Plate VC). The early growth of the plants was considerably decreased. In the course of the growing period the leaf colour as well as the development of the deficient plants improved so much that the differences in the foliage of control plants and those

TABLE XII

Effect of molybdenum on yield of sugar beet *) grown on an ironstone-containing peaty soil of pH 5.8 (Exp. 1297–1, 1953)

$Na_2MoO_4.2H_2O$ kg per ha	Sugar beet, q per ha		Sugar content of beet
	Beet	Foliage	
0	377 ± 26	620 ± 41	15.2
0.5	492 **)	657 **)	16.8
1	468 **)	594 **)	
2	504 **)	636 **)	
4	399 **)	528 **)	

*) Manuring: 160 kg N per ha as $Ca(NO_3)_2$, 100 kg P_2O_5 per ha as $CaHPO_4$, 300 kg K_2O per ha as K_2SO_4, 20 kg MgO per ha as $MgSO_4.7H_2O$.
**) Single values.

Plate V. A: Radish, B: spinach, grown on a molybdenum-deficient soil. *Left* and *centre*. without added molybdenum; *right*: dressed with 2 kg of $Na_2MoO_4.2H_2O$ per ha. C: Sugarbeet. *Left*: no Mo; *right*: 1 mg $Na_2MoO_4.2H_2O$ per pot. D: Cauliflower. From *left* to *right*: 0 Mo, 0 N; 0 Mo, + KNO_3 + Mo, 0 N and + Mo, + KNO_3. E: Cauliflower. From *left* to *right*: 0 Mo, KNO_3; 0 Mo, NH_4NO_3; + Mo, KNO_3 and + Mo, NH_4NO_3.

Plate VI. Effect of added manganese sulphate on response of cauliflower to molybdenum. From *left* to *right*: 0, 200, 500, 1000 and 2000 mg of $MnSO_4.4H_4O$ per pot. A: no molybdenum; B: 100, C: 200, D: 1000 and E: 5000 µg $Na_2MoO_4.2H_2O$ per pot.

dressed with molybdate disappeared. The differences in yield may be rather considerable, however, as will be seen from the results of Field experiment 1297–1 (Table XII). In addition to an increased weight of roots, the sugar content was also clearly higher in the case of molybdenum treatment. 4.0 kg of molybdate was apparently too high for this crop. The harmful effect of relatively small amounts of molybdenum on sugar beet was also observed in subsequent pot experiments.

Potato. On some soils potato showed pale green leaves as contrasted to the dark green leaves of the plants treated with a few kg of molybdate per ha. Yield of tubers of the latter plants was only slightly higher, however, as may be seen from Table XIII. In a number of cases in which cauliflower and leguminous crops gave a pronounced response to added molybdenum, potato was not affected. In one case a decrease in yield was obtained by amounts higher than 0.5 kg molybdate per ha (Table XIII).

TABLE XIII

Effect of molybdenum dressings on yield of potato grown on ironstone-containing soils *)

$Na_2MoO_4.2H_2O$ kg per ha	Yield of tubers, q per ha			
	1297–1 (1952)		1297–4 (1953)	
0	434 ± 9		385 ± 14	
0.5	510 **)	482 ± 19	420 **)	326 ± 4
1	453 **)		327 **)	
2	444 **)		318 **)	
4	519 **)		333 **)	

*) Manuring: 160 kg N per ha as $Ca(NO_3)_2$, 100 kg P_2O_5 per ha as $CaHPO_4$, 300 kg K_2O per ha as K_2SO_4, 20 kg MgO per ha as $MgSO_4.7H_2O$.

**) Single values.

Tomato growing on ironstone-containing soil had mottled leaves when no molybdenum had been applied. The mottling soon became worse so that the whole plant developed a yellow colour. Necrotic spots were seen in the yellow tissue and the leaflets showed an involution (cf Plate VIII). Many older leaves died. These symptoms were similar to those observed in molybdenum-deficient culture solutions [33]).

Cereals. Wheat and oats were grown on Exp. field 1297–1 in 1952 and 1953; barley was grown in 1952 only. Commercial seed was used in 1952. In order to eliminate the effect of the molyb-

denum contained in the grain, seed of the first year's wheat crop was sown in the second year.

A very pronounced effect of added molybdenum was observed in wheat, particularly in the second year. The young plants were highly chlorotic and made a much poorer growth than those dressed with molybdate. The colour differences almost disappeared at a later stage but ear emergence was much retarded in the molybdenum-deficient plants. Deaf ears occurred rather frequently at harvest time and ripening of the crop was delayed. The yield of the molybdenum-deficient plants was badly affected (Table XIV) and the kernels had a very shrivelled appearance. In this respect molybdenum deficiency symptoms resembled those of copper deficiency [32]).

In the case of oats, clear differences in leaf colour and slight differences in development of the plants were observed in the first part of the growing period. The differences in yield were insignificant. Barley did not respond to added molybdenum.

TABLE XIV

Effect of molybdenum dressings on yield, q (100 kg) per ha, of wheat *) (Exp. 1297–1)

$Na_2MoO_4.2H_2O$ kg per ha	Wheat, 1952		Wheat, 1953	
	Grain	Straw	Grain	Straw
0	24.3 ± 2.2	50.0 ± 0.9	22.1 ± 1.8	68.7 ± 2.8
0.5	21.6 **)	51.3 **)	—	—
1	42.3 **)	83.7 **)	—	—
2	36.0 **)	63.9 **)	35.3 ± 4.3	90.6 ± 7.8
4	36.9 **)	62.1 **)	34.9 ± 3.6	76.7 ± 2.7

*) Manuring: 120 kg N per ha as calcium nitrate, 100 kg P_2O_5 per ha as $CaHPO_4$, 20 kg MgO per ha as $MgSO_4.7H_2O$, 50 kg $CuSO_4.5H_2O$ per ha.

**) Single values.

POT EXPERIMENTS WITH VERY ACID SOILS

According to L e w i s [27]) molybdenum is more available to plants in alkaline soils than in acid ones. This was concluded from experiments with pastures high in available molybdenum. On these soils, the so-called "teart pastures" of Somerset, very high concentrations of molybdenum were found in the plants. Cattle fed with this pasturage very often suffered from molybdenum poisoning. Acidification of the soil resulted in a considerably lower molybdenum uptake by the plants. From the results of his experiments L e w i s concluded that lowering of the pH decreased the availability of soil molybdenum.

Oertel *et al.* [41]) and Anderson and Moye [4]) studied the effect of liming of molybdenum-deficient Australian soils. In agreement with the experiments of Lewis it was found that no molybdenum deficiency of the plants growing on these soils occurred when the latter had been limed heavily. From these results, as well as from those of earlier experiments, Oertel *et al.* and Anderson and Moye concluded that a rise of pH favours the availability of soil molybdenum. Neither Anderson and Moye nor Oertel *c.s.* and Lewis have determined plant-available molybdenum in soils treated with acids or lime. Their conclusion as to the effect of pH on availability of soil molybdenum is derived from the state of health and the molybdenum content of plants growing in the respective soils.

Although from a chemical point of view it looks probable that a rise of soil pH will promote the solubility of molybdenum, it might be possible that the beneficial effect of liming on molybdenum uptake by the plants is due at least partly to an indirect effect, viz. the removal of substances which inhibit the uptake of soil molybdenum by the plant roots. That this latter effect under certain circumstances would be of considerable importance may be concluded from the following experiments.

Cauliflower plants were cultivated on a number of very acid soils in Mitscherlich pots. These soils came from fields where experiments on liming on different soil types had been laid out by members of this station a considerable number of years ago.

The basic dressing consisted of K_2SO_4, 2 g; $CaHPO_4$, 3 g; $MgSO_4.7H_2O$, 0.75 g; $Ca(NO_3)_2$, 0.5 g (leguminous plants) and 4.25 g (cauliflower and potato), while molybdenum was added in amounts of 10 mg $Na_2MoO_4.2H_2O$ per pot to a number of pots. Control pots received 10 mg of $Na_2CO_3.10H_2O$.

As will be seen from the data of Table XV the cauliflower plants grown on most of these soils responded to added sodium molybdate. The untreated plants showed a severe mottling and cupping which was absent in the presence of added molybdenum. At the time of thinning the plants, the differences in dry weight per plant in most cases were relatively high. On four of these soils the cauliflower plants at a later stage showed typical symptoms of whiptail (see Plate IIIB).

White clover and potato which were sown simultaneously in parallel pots did not respond to molybdenum when growing in these soils, with the exception of white clover on soil no 6 which, however, was an ironstone-containing peaty soil. In spite of the very low pH values of these soils, white clover was provided with many nodules

TABLE XV

Effect of molybdenum on cauliflower, white clover, lucerne and potato, grown on acid soils (pot experiment) *)

Soil type	pH	Cauliflower			Lucerne		White clover		Potato	
		Dry weight of young plants, mg per plant		Whip-tail **)	Dry weight, g per pot		Dry weight, g per pot		Tubers, g per pot	
		0 Mo	10 mg $Na_2MoO_4.2H_2O$		0 Mo	10 mg $Na_2MoO_4.2H_2O$	0 Mo	10 mg $Na_2MoO_4.2H_2O$	0 Mo	10 mg $Na_2MoO_4.2H_2O$
peaty, Exp. 836	3.5	75	150	+	4.3	5.8	28.8	25.3	260	260
sandy, Exp. 837	3.7	143	170	—	10.4	12.0	25.5	24.2	228	240
low moor peat, Exp. 200	4.0	171	395	+	10.5	12.8	23.2	25.5	245	243
reclaimed low moor peat, Exp. 119	4.1	153	587	—	16.0	18.8	30.6	30.8	233	230
sandy, Exp. 10	4.0	554	515	—	13.3	12.4	34.0	28.6	240	253
sandy, Exp. 1297–4	5.6	70	525	+	12.5	19.9	27.9	34.5	210	203
sandy, Exp. 263	3.7	647	750	—	22.6	22.2	33.9	35.3	160	173
peaty, Exp. 24	4.0	450	546	—	9.4	11.4	25.1	22.6	178	195
reclaimed low moor peat, Exp. 13	3.9	50	108	+	3.9	3.2	18.7	18.6	203	210

*) Averages of duplicate values.

TABLE XVI

Available molybdenum (*Asp. niger*) of various soils in relation to response of higher plants to added molybdenum

Soil type	pH	Available Mo (*Asp. niger*), ppm **)	White clover, mg N in crop per pot		Cauliflower, response to added Mo
			0 Mo	+ Mo	
low moor peat (Fe) *)	5.5	0.003	43.9	110.8	—
sandy (Fe)	6.1	0.005	44.0	92.4	strong
low moor peat (Fe)	6.0	0.006	6.6	40.4	—
sandy (Fe)	5.5	0.007	29.0	114.0	—
sandy (Fe)	4.6	0.007	—	—	—
sandy (Fe)	5.7	0.035	40.0	109.9	—
sandy	5.3	0.044	18.0	58.3	—
sandy (Fe)	5.4	0.044	14.5	68.8	strong
sandy	5.6	0.052	50.8	76.9	slight
sandy (Fe)	5.8	0.063	63.0	126.4	strong
sandy loam	—	0.100	no response		no response
sandy, normal arable soil	5.6	0.125	—	—	—
low moor peat (Fe)	5.8	0.153	51.6	158.7	strong
sandy loam, fertile garden soil	7.0	>0.4	—	—	—
sandy (Fe), Tasmania (Kinburn)	6.2	0.017	110.5 †)	382.0 †)	—
sandy (Fe), South Australia (Houghton)	5.4	0.002	93.4 †)	170.0 †)	—
peaty, Exp. field 119	4.1	0.008	no response		strong
sandy, ,, ,, 10	4.0	0.046	,, ,,		slight
peaty, ,, ,, 24	4.0	0.093	,, ,,		no response
peaty, ,, ,, 13	3.9	0.112	,, ,,		moderate
peaty, ,, ,, 836	3.5	>0.2	,, ,,		strong
sandy, ,, ,, 263	3.7	>0.2	,, ,,		no symptoms
sandy, ,, ,, 837	3.7	>0.2	,, ,,		no symptoms
low moor peat, Exp. field 200	4.0	>0.2	,, ,,		strong

*) (Fe) = rich in ironstone. **) Averages of triplicate values.

†) Large vessels used.

which were able to supply the plants with adequate amounts of nitrogen both in the absence and presence of added molybdenum. Apparently the clover plants succeeded in absorbing adequate amounts of molybdenum from these acid soils to enable the nodules to fix atmospheric nitrogen. In these experiments the difficulty of inoculating the clover roots with *Rhizobium* under acid soil conditions was overcome by mixing the clover seed with a mixture of *Rhizobium* and molybdenum-deficient peaty soil of pH 5.8.

In the case of lucerne which was also grown in this experiment,

the inoculation method gave less favourable results so that in a number of soils the plants were free from nodules both in the absence and presence of added molybdenum. Lucerne responded slightly to added molybdenum in some cases.

Available molybdenum in the acid soils used in this experiment as well as in a number of other soils was estimated by the *Aspergillus niger* assay method. As will be seen from the results of these estimations (Table XVI), the ironstone-containing soils generally are very low in available molybdenum. Relatively high values were found, however, in a number of acid soils on which the cauliflower plants showed severe symptoms of molybdenum deficiency. Apparently cauliflower is unable to absorb the available molybdenum present from these soils owing to the interaction of some unknown factors. Since acid soils are rich in plant-available manganese it was suggested that excess of manganese might be responsible for the poor uptake of molybdenum by cauliflower plants. To verify this assumption a number of pot experiments were carried out in which molybdenum and manganese in different amounts were added to molybdenum-deficient soils. A description of these experiments will be given in the following sections. Manganese was determined in the clover plants grown on the acid soils. The results of these determinations will be discussed below.

EFFECT OF VARIOUS SOIL CONDITIONS ON RESPONSE TO ADDED MOLYBDENUM OF GREEN PLANTS

When a comparison is made of plants growing on molybdenum-deficient soil with those in culture solutions purified rigorously from molybdenum impurities, it will be found that in the solutions much smaller additions of molybdenum are effective than in the soil. The following pot experiment has been carried out to determine whether this difference is associated with the uptake of some toxic substance from the soil or is attributable to the presence in the soil of substances fixing molybdenum or interfering with its uptake by the plant roots, as has been suggested in the foregoing section. Glass jars of 600 ml capacity were filled in different ways with molybdenum-deficient soil of pH 5.8 and purified quartz sand:

a) Molybdenum-deficient soil only. Sodium molybdate was

supplied in amounts of 25 and 500 μg respectively, whilst some pots remained without added molybdenum.

b) One half of each pot was filled with molybdenum-deficient soil, the other half with quartz sand. In order to prevent mixing of soil and sand, a glass plate was placed in the jars which was removed after the filling had been completed. Cauliflower seeds were placed on the border line between soil and sand. This way of planting enabled the plants to grow with part of their roots in the sand and with the others in the soil. Both halves of the pot were supplied with a complete nutrient solution lacking molybdenum. One set of jars remained without added molybdenum. A second received 25 and 500 μg respectively of Na_2MoO_4 in the sand and in a third set these amounts were mixed through the soil.

c) The molybdenum-deficient soil and the sand as used under *b*) were thoroughly mixed before placing them in the jars.

d) Slightly molybdenum-deficient soil (pH 4.0) only, with or without 500 μg of sodium molybdate.

e) One half of the jars was filled with the slightly deficient soil, the other half with the severely deficient one.

f) The same soils as mentioned under *e*) but mixed thoroughly before filling the jars.

Cauliflower plants growing in the *a*-pots required the highest amount of molybdate (500 μg) to give healthy plants. Supplied with 25 μg they still were severely deficient (Table XVII). The plants growing with part of their roots in the soil and with the others in the sand (*b*) were completely healthy, even in the absence of added molybdenum. The small amount of molybdenum, which in other experiments was shown to be present as a contamination in the purified quartz sand, sufficed for a normal plant growth. Where sand and soil had been mixed, however, (*c*), the symptoms of molybdenum deficiency were quite similar to those of plants, growing in only soil (*a*). Supplied with 25 μg, the plants of the (*c*) pots gave higher yields than without added molybdenum but they still showed typical symptoms of molybdenum deficiency.

A filling of separate deficient and a slightly deficient soil (*e*) reacted like the latter whereas a mixture of them (*f*) reacted like the severely deficient soil.

The results of this experiment show that the molybdenum-deficient soil either was able to fix added molybdenum or contained

some substance which inhibits molybdenum uptake by the cauliflower plants.

TABLE XVII

Effect of soil and filling of pots on response of cauliflower to added molybdenum (dry weight of plants in mg) *)

Soil and filling of pots	$Na_2MoO_4.2H_2O$, μg per pot		
	0	25	500
a. Mo-deficient soil	240 (sev) **)	343 (sev)	1195 (n)
b. ½ Mo-def. soil, ½ sand (separately filled)	1382 (n)	1285 †) (n) 1133 ††) (n)	1097 †) (n) 1375 ††) (n)
c. ½ Mo-def. soil, ½ sand (mixed)	175 (sev)	593 (mod)	715 (n)
d. Slightly Mo-def. soil	372 (sl)	—	497 (n)
e. ½ slightly Mo-def. soil, ½ Mo-def. soil, (separately filled)	407 (sl)	—	—
f. ½ slightly Mo-def. soil, ½ Mo-def. soil, (mixed)	125 (sev)	—	635 (n)

*) Averages of duplicate values.

**) sev = severe symptoms of molybdenum deficiency, mod = moderate, sl = slight symptoms, n = normal plants.

†) Molybdenum added to the sand half.

††) Molybdenum added to the soil half.

Effect of soil manganese on response to molybdenum of green plants

In order to elucidate the effect of manganese on response to added molybdate of green plants, growing on molybdenum-deficient soil, a pot experiment was carried out with two soils, an ironstone-containing peaty soil of pH 5.8 from Field exp. 1297–1 and a similar sandy soil of pH 5.6 from Field exp. 1297–4. Neubauer glass jars of 1 l capacity containing approximately 1 kg of soil were used for growing the cauliflower plants (10 plants per pot). As a basic dressing the following salts were added per pot: KNO_3 1 g, K_2HPO_4 0.4 g, $MgSO_4.7H_2O$ 0.1 g, $Na_2CO_3.10H_2O$ 1.2 mg. Molybdenum was applied in amounts of 0.1, 0.2, 1 and 5 mg $Na_2MoO_4.2H_2O$, manganese in amounts of 0, 200, 500, 1000 and 2000 mg $MnSO_4.4H_2O$. In the case of the peaty soil glass jars of 0.6 l capacity were employed to contain approximately 0.5 kg of soil and the amounts of added basic nutrients, manganese and molybdate were proportionally reduced.

Without applied Mo and Mn the cauliflower plants showed severe symptoms of molybdenum deficiency which were absent when

100 μg of $Na_2MoO_4.2H_2O$ had been added. When provided with 200 mg of $MnSO_4$ in addition the beneficial effect of this dose of sodium molybdate was completely eliminated. Higher amounts of molybdate in the presence of 200 mg $MnSO_4$ gave normal plants. The higher the dose of manganese sulphate applied, the higher the amount of molybdate required for healthy plant growth. Plate VI and Fig. 1 give the results of the sandy soil, Table XVIII those of the peaty soil.

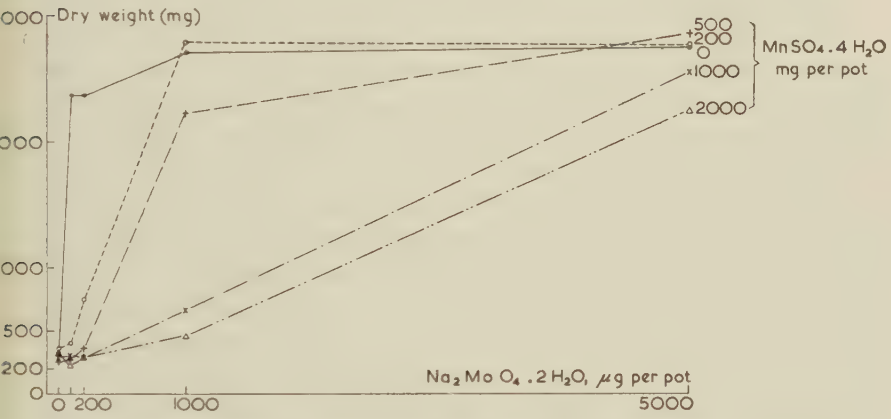

Fig. 1. Effect of added manganese sulphate on response of cauliflower to molybdenum.

TABLE XVIII

Effect of various concentrations of manganese sulphate on response of cauliflower to molybdenum (dry weight of young plants, g) *)

$Na_2MoO_4.2H_2O$, μg per pot	$MnSO_4.4H_2O$, mg per pot			
	0	100	250	500
0	0.45	0.40	0.25	0.35
25	1.85	0.60	0.50	0.45
50	2.75	0.50	0.65	0.35
100	3.45	2.75	0.50	0.45
500	3.85	3.70	3.10	2.35
2500	3.80	2.95	3.10	3.60

*) Single values, no Mn in triplicate.

From the results of these pot experiments it can be concluded that the harmful effect of manganese sulphate on cauliflower plants growing on molybdenum-deficient soil, treated with a moderate amount of molybdate, must be largely attributed to its interference with molybdenum nutrition. This can be concluded from the fact

that the symptoms of molybdenum deficiency which occur in the plants without added manganese and molybdenum are identical with those of plants treated with a moderate amount of molybdate and excess of manganese sulphate and from the fact that added molybdate is able to eliminate the effect of excessive $MnSO_4$.

In addition to this effect of interfering in molybdenum nutrition, manganese sulphate may bring about symptoms of manganese toxicity in cauliflower. The latter occurs on soils relatively rich in available molybdenum and high in manganese but low in pH so that high concentrations of available manganese may persist in the soil. On such soils the amount of manganese required for causing Mo-deficiency is apparently higher than the amount required for bringing about the symptoms of Mn-toxicity. The following pot experiment gives an example of such conditions.

The soil used was a mixture of a sandy soil of pH 3.7 and a peaty soil of pH 4.0. It was contained in Neubauer glass jars of 1 l capacity. The basic dressing consisted of KNO_3 1 g, K_2HPO_4 0.4 g, $MgSO_4.7H_2O$ 0.1 g and $Na_2CO_3.10H_2O$ 1.2 mg. $MnSO_4.4H_2O$ was added in amounts of 500, 1000 and 2000 mg per pot, and molybdenum in amounts of 1, 5, 10 and 20 mg $Na_2MoO_4.2H_2O$ per pot. In the absence of added $MnSO_4$ a slight response to molybdenum was observed (Table XIX). This was also the case when manganese sulphate was added, but here the development of the molybdenum-treated plants was also much affected. Independently of the amount of molybdenum added, manganese toxicity symptoms occurred, particularly in the plants treated with 1000 and 2000 mg of $MnSO_4$. Plants treated with 500 mg $MnSO_4$ were slightly mottled in the absence of added molybdenum. This mottling never was found in molybdenum-treated plants.

TABLE XIX

Effect of various concentrations of $MnSO_4$ and Na_2MoO_4 on yield of cauliflower plants, grown on an acid soil, relatively rich in available molybdenum (dry weight in g)*)

$Na_2MoO_4.2H_2O$, mg per pot	$MnSO_4.4H_2O$, mg per pot			
	0	500	1000	2000
0	2.12	1.48	1.10	0.27
1	2.52	1.35	1.39	0.52
5	2.54	1.85	1.38	0.41
10	2.64	1.45	1.34	0.51
20	2.38	1.88	1.10	0.55

*) Single values.

Although the symptoms of molybdenum deficiency in cauliflower resemble those of manganese toxicity, they are not identical. Both manganese toxicity and molybdenum deficiency bring about a cupping of the laminae, but the upward curling in the former case is confined to the margin of the leaves whereas in the case of molybdenum deficiency a much greater part of the laminae takes part in the cupping. Mo-deficient leaf tissue is mottled or chlorotic as contrasted to the dark green colour of leaves with manganese toxicity. In addition manganese toxicity is unaffected by molybdenum treatment.

Pot experiments with various Mn-compounds and with various sulphates. In order to know whether the effect of manganese sulphate on molybdenum nutrition of cauliflower plants is attributable to the Mn-ions or to the sulphate, pot experiments with cauliflower plants provided with various Mn-compounds, $MnSO_4$, $Mn(NO_3)_2$ and Mn-citrate respectively, and with various sulphates, K-, Na- and Ca-sulphates respectively, in combination with various doses of sodium molybdate have been carried out. The soil used in this experiment was the ironstone-containing peaty soil on which in previous experiments cauliflower was found to give severe symptoms of molybdenum deficiency. The plants were grown in Neubauer jars which contained approximately $\frac{1}{2}$ kg of soil. The basic dressing consisted of 0.5 g of KNO_3, 0.2 g of K_2HPO_4, 0.05 g of $MgSO_4.7H_2O$ and 0.5 mg of $Na_2CO_3.10H_2O$. Molybdenum was supplied in amounts of 5%, 10, 500 and 2500 *mg* of $Na_2MoO_4.2H_2O$, manganese and sulphate in amounts equivalent to 100, 250, 500 and 1000 mg of $MnSO_4.4H_2O$ per pot.

The results of this experiment show that the antagonistic effect of $MnSO_4$ on molybdenum activity is partly due to the SO_4-ions, partly to the Mn-ions (Table XX, Fig. 2). This may be concluded from the inhibiting effect of potassium, sodium and calcium sulphates on molybdenum activity. The effect of calcium sulphate was found to be more pronounced than that of the other sulphates, possibly due to a slight lowering of pH upon treatment with the former salt as a result of which the concentration of available manganese may have been increased.

The inhibiting effect of Mn-ions may be seen particularly clearly when the highest amount of $Mn(NO_3)_2$ and an equivalent amount of

TABLE XX

Effect of $MnSO_4$, $Mn(NO_3)_2$, Mn-citrate, Na_2SO_4, K_2SO_4 and $CaSO_4$ on response of cauliflower plants to molybdenum dressings (dry weight of plants, g per pot) *)

Treatment	$Na_2MoO_4.2H_2O$, μg per pot				
	0	50	100	500	2500
Control	370	1056	1205	1795	2153
100 mg $MnSO_4.4H_2O$	430	565	525	1190	1910
250 ,, ,,	405	505	410	1125	2025
500 ,, ,,	430	455	470	1115	1850
1000 ,, ,,	330	350	405	640	1715
80 mg $Mn(NO_3)_2$ **)	375	1140	1370	1545	1695
200 ,, ,,	380	1005	1050	1880	2185
400 ,, ,,	295	730	975	1480	1550
800 ,, ,,	420	575	670	1320	1765
909 mg KNO_3	255	665	1865	2005	1975
81.2 mg Mn-citrate	415	540	1405	1655	1680
203 ,, ,,	460	605	665	2020	1550
406 ,, ,,	475	730	950	1595	1375
812 ,, ,,	380	435	565	1265	1250
144.2 mg $Na_2SO_4.10H_2O$	400	640	885	1685	2085
360.5 ,, ,,	415	430	485	1710	1675
721 ,, ,,	340	385	485	(880)	1950
1442 ,, ,,	365	330	450	1480	1950
77.8 mg K_2SO_4	375	410	600	1565	2110
194.5 ,, ,,	415	490	715	1485	1835
389 ,, ,,	305	430	575	1285	1875
778 ,, ,,	410	580	500	1235	1965
77.2 mg $CaSO_4.2H_2O$	520	625	1080	1795	1630
193 ,, ,,	580	585	465	1420	1825
386 ,, ,,	450	345	400	1135	1775
772 ,, ,,	375	460	470	640	1740

*) Single values, control in duplicate. Sown Sept. 1, harvested Oct. 24, '52.
**) $Mn(NO_3)_2$ was substituted for KNO_3.

KNO_3 are compared at 100 and 500 μg of Na_2MoO_4. The former brought about severe molybdenum deficiency whereas KNO_3 gave quite normal plants.

To determine whether the effect of manganese and the sulphates was due to a reduced molybdenum uptake, molybdenum determinations were carried out in the leaves of the cauliflower plants. For these estimations the *Aspergillus* technique was employed. The

results of these experiments are given in Table XXI. It will be seen that the molybdenum contents of plants treated with manganese compounds or with sulphates were considerably lower than those of the control plants. The manganese effect is shown particularly clearly when plants treated with 800 mg of $Mn(NO_3)_2$ and those with an equivalent amount of KNO_3 are compared. The latter plants had from 4 to 5 times as much molybdenum in their tissues as those dressed with $Mn(NO_3)_2$.

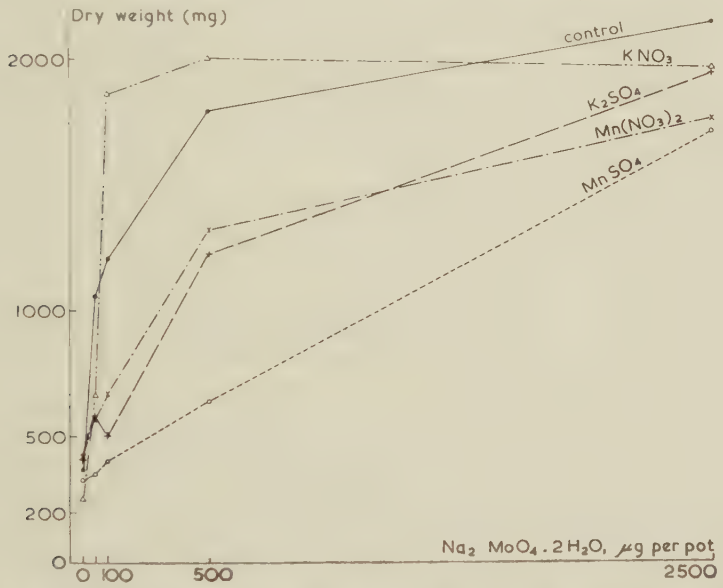

Fig. 2. Effect of $MnSO_4$, $Mn(NO_3)_2$, KNO_3 and K_2SO_4 on response of cauliflower to molybdenum.

In lettuce growing on molybdenum-deficient soil a moderate effect of manganese sulphate on molybdenum uptake was found. In the case of white clover, tomato and spinach the response of the plants to added molybdenum was practically not affected by manganese sulphate.

Nutrient-solution experiments. Although the Mn-Mo-interrelationship was found consistently in cauliflower growing in molybdenum-deficient soil, the author has been as yet unable to obtain this effect in nutrient solution. In an experiment with the latter the cauliflower plants were cultivated in glass jars

TABLE XXI

Effect of $MnSO_4$, $MnNO_3$ and K_2SO_4 on molybdenum uptake by cauliflower plants, grown in molybdenum-deficient soil, at increasing amounts of Na_2MoO_4. (Mo in ppm of dry matter of leaves and stem respectively) *)

Treatment	$Na_2MoO_4.2H_2O$, μg per pot									
	0		50		100		500		2500	
	leaf	stem	leaf	stem	leaf	stem	leaf	stem	leaf	stem
Control	0.06	0.05	0.13	0.09	0.17	0.10	0.11	0.37	0.81	0.37
100 mg $MnSO_4.4H_2O$	—	—	—	—	—	—	0.04	—	—	—
250 ,, ,,	—	—	—		—	—	0.05	—	—	—
500 ,, ,,	—	—	—	—	—	—	0.06	—	—	—
1000 ,, ,,	—	—	—	—	—	—	0.06	—	—	—
80 mg $Mn(NO_3)_2$	0.02	0.03	—	—	—	—	0.10	0.05	1.27	0.46
200 ,, ,,	0.02	0.01	—	—	—	—	0.15	0.23	0.48	0.43
400 ,, ,,	—	0.02	—	—	—	—	0.05	0.06	0.32	0.24
800 ,, ,,	0.01	0.03	—	—	—	—	0.03	0.04	0.25	0.14
909 mg KNO_3	0.05	0.05	—	—	—	—	0.15	0.17	0.91	0.46
77.8 mg K_2SO_4	—	—	—	—	—	—	0.06	—	—	—
194.5 ,, ,,	—	—	—	—	—	—	0.05	—	—	—
389 ,, ,,	—	—	—	—	—	—	0.05	—	—	—
778 ,, ,,	—	—	—	—	—	—	0.07	—	—	—

*) Averages of triplicate values.

of 0.6 l capacity containing a solution of the following composition: KNO_3 0.75 g, $NaH_2PO_4.2H_2O$ 0.5 g, $MgSO_4.7H_2O$ 0.2 g, $CaSO_4.2H_2O$ 0.15 g, $Fe_2(NH_4)_2(SO_4)_4.24H_2O$ 40 mg, $CuSO_4.5H_2O$ 0.5 mg, $ZnSO_4.7H_2O$ 0.5 mg, H_3BO_3 0.5 mg per l of glass-distilled H_2O at a pH of approximately 6.5. Initially Mn was added in amounts of 0, 0.5 and 10 mg $MnSO_4.4H_2O$ and Mo in amounts of 0, 1 and 100 μg $Na_2MoO_4.2H_2O$ per l. When symptoms of Mn-deficiency appeared in the nil-Mn cultures 0.1 mg of manganese sulphate was added per pot. Furthermore molybdenum of the pots with 1 μg was increased to 5 μg $Na_2MoO_4.2H_2O$ and the iron concentration of all pots was doubled.

Moderate symptoms of molybdenum deficiency were observed in the plants growing in this medium without added molybdenum, viz., a slight mottling, a poorer growth than that of the molybdenum-supplied plants and in a later stage no heart formation. Chlorosis and poor growth were more pronounced in the case of low Mn and less pronounced in high Mn plants than in those with moderate Mn. Plants supplied with high molybdenum made less growth

than those with moderate Mo. This was particularly true in the case of high manganese. The results of this experiment show that the manganese-molybdenum antagonism is apparently confined to certain plant species growing under soil conditions.

A molybdenum-manganese antagonism has been observed by Millikan in nutrient-solution experiments with flax [30]). The harmful effect of excessive amounts of manganese on growth of this plant was found to be strongly reduced by molybdate. The amounts of molybdenum required for exerting this effect were more than a hundred times higher than those used in the present investigation so that it is highly questionable whether the symptoms of manganese toxicity described in Millikan's experiment were identical with those of molybdenum deficiency. In contrast to Millikan's results with flax, Hewitt found that relatively high amounts of molybdenum may accentuate chlorosis caused by excess of manganese in sand cultures of sugar beet [19]). In agreement with the latter results, Warington [49]) showed that molybdenum in a concentration similar to the one used by Millikan may intensify the chlorosis induced by manganese excess in flax and soybean. Löhnis [28]) was unable to show any effect of molybdenum on manganese toxicity of flax growing in a nutrient solution. Hannay and Street [18]) have studied manganese-molybdenum relationships in excised roots of tomato plants. Molybdenum deficiency was found to be markedly alleviated by additions of manganese at concentrations which were clearly toxic in the presence of added molybdenum.

Anderson and Spencer [5]) found a decreased molybdenum content in subterranean clover and flax growing in Australian soils supplied with excessive $MnSO_4$. This effect may have been due to both Mn^{++} and SO_4^{--}. Stout *et al.* [46]) have made an extensive investigation of the influence of sulphates on uptake of molybdenum by pea and tomato plants. Addition of K_2SO_4 or $CaSO_4$ to culture solutions as well as to soils resulted in considerably reduced molybdenum contents of these plants. Similar results have been obtained by Plant [43]) in field experiments with cauliflower plants. Retention or excretion of molybdenum by sheep was found by Dick [13]) to be appreciably affected by the amount of inorganic sulphate in the diet. At low sulphate levels molybdenum was absorbed from the food and accumulated in the blood and tissues. At higher sulphate intake appreciably more molybdenum was excreted in the urine and the accumulation in the blood did not occur.

Effect of phosphate on response to molybdenum of green plants and Asp. niger

Molybdenum-deficient soils were frequently found to be poor in available phosphorus. This may be concluded from Table XXII in which the results of a pot experiment with white clover are given.

In this experiment the response of the clover to molybdenum has been studied at three levels of phosphorus supply, viz. 0, 100 and 400 mg of K_2HPO_4 per pot, containing approximately $\frac{1}{2}$ kg of soil. The basic dressing of these plants consisted of K_2SO_4 (200, 100 and 0 mg respectively for the three P levels), $MgSO_4.7H_2O$ 50 mg, $Na_2CO_3.10H_2O$ 0.12 mg (added to the 0 Mo-plants only). A number of pots were supplied with 0.5 mg $Na_2MoO_4.2H_2O$.

TABLE XXII

Effect of molybdate and phosphate dressings on yield *) of white clover, g dry weight per pot, grown on different soils

Soil	No added molybdenum			0.5 mg of $Na_2MoO_4.2H_2O$ per pot		
	no added P	100 mg K_2HPO_4	400 mg K_2HPO_4	no added P	100 mg K_2HPO_4	400 mg K_2HPO_4
1	3.47	4.32	5.94	3.83	4.64	7.37
2	0.52	1.34	3.35	0.41	2.05	4.82
3	1.94	2.54	1.97	1.94	2.71	2.82
4	2.09	3.13	4.73	3.47	5.23	8.12
12	1.31	2.11	3.43	2.05	2.90	4.80
14	1.61	2.82	4.33	1.75	3.86	6.24
16	1.62	4.13	6.08	2.72	4.13	8.21
17	0.31	0.34	0.99	0.60	0.86	1.45
19	2.96	3.37	5.21	3.50	4.45	5.59
20	1.08	1.16	2.20	1.61	2.33	4.83
22	1.34	1.76	4.04	1.03	1.93	4.57
69	0.95	1.40	4.58	6.20	6.43	8.54

*) Average values of duplicate pots.

It will be seen that generally the phosphate dressings did not affect the response of the plants to molybdenum. Merely in the case of soil 69 there was a clear lessening of molybdenum deficiency due to phosphate dressings.

The occurrence of molybdenum and phosphate deficiencies in the same soils suggests that the same soil conditions are responsible for rendering these nutrients unavailable to the plants. Since molybdenum-deficient soils frequently are characterized by a high content of ironstone, it is obvious that iron has something to do with this process. In field trials on ironstone-containing soils a reduction in molybdenum effect in the second year after the application of molybdenum was observed. This reduction was rather small in the case of cabbage so that a dose of 1 kg $Na_2MoO_4.2H_2O$ per ha gave a considerable after-effect (see Table VIII). In cauli-

flower, however, the direct effect of added molybdenum was of much more importance than the after-effect.

The beneficial effect of added phosphate on molybdenum supply of the plants on soil 69 might be attributed to a solubilization of soil molybdenum by added phosphate or to a stimulation of its uptake by the plants.

The effect of added phosphate on molybdenum uptake by white clover growing on soil 69 is in agreement with the results of a pot experiment with cauliflower in which in addition to the basic dressing of 200 mg KH_2PO_4, 200, 500, 1000 and 2000 mg respectively of this compound had been added per pot (containing $\frac{1}{2}$ kg of molybdenum-deficient soil). Without added molybdenum practically no effect of phosphate level on the molybdenum-deficiency symptoms was observed. In the case of plants treated with 50 μg and particularly in those treated with 100 μg of sodium molybdate, however, phosphate exerted a beneficial effect on the appearance of the plants apparently due to an increased molybdenum supply. The latter effect may be seen in Table XXIII in which the yields and the molybdenum contents of the cauliflower leaves of this experiment are given.

TABLE XXIII

Effect of phosphate and molybdate dressings on yield, g dry weight per pot, and molybdenum content, ppm of dry matter, of cauliflower plants grown on an iron-stone-containing peaty soil

KH_2PO_4 mg per pot	$Na_2MoO_4.2H_2O$, μg per pot							
	0		50		100		2500	
	Yield, g	Mo, ppm	Yield, g	Mo, ppm	Yield, g	Mo, ppm	Yield, g	Mo, ppm
200	370	0.06	1076	0.13	2251	0.17	2150	0.81
400	425	—	1048	—	1592	—	1599	—
700	470	—	1181	—	1517	—	1830	—
1200	425	—	1070	0.05 *) 0.09 **)	1327	—	1977	1.70 *) 1.50 **)
2200	405	—	940	0.07 *) 0.18 **)	1351	—	1850	—

*) veinal tissue.

**) interveinal tissue.

Observations which are in agreement with the results of these pot experiments have been made by the author on two experimental fields with white clover and cabbage in England and Wales during the summer

of 1953. The white clover plants growing on a phosphate-deficient soil showed a yellow-green leaf colour as contrasted to the dark green of the phosphate-treated plants. The former plants contained numerous small yellow root nodules which resembled those of molybdenum-deficient plants and which apparently were unable to fix atmospheric nitrogen. The plants treated with phosphate had normal pink nodules.

In the case of cabbage severe symptoms of molybdenum deficiency occurred on the plots without added phosphate. Dressed with phosphate no molybdenum deficiency was seen. Although no molybdenum analyses of the phosphatic fertilisers used in these field experiments are available, it is highly improbable that the molybdenum impurities of the phosphates used could have been responsible for the beneficial effect of the phosphate dressings. Both British soils have been tested in the author's laboratory for available molybdenum using the *Aspergillus niger* technique. Furthermore cauliflower was grown on these soils under greenhouse conditions both with and without added molybdenum. Severe symptoms of molybdenum deficiency occurred in the cauliflower when no molybdenum had been added. In the *Aspergillus* tests relatively high values for available soil molybdenum were found, viz. > 0.2 ppm.

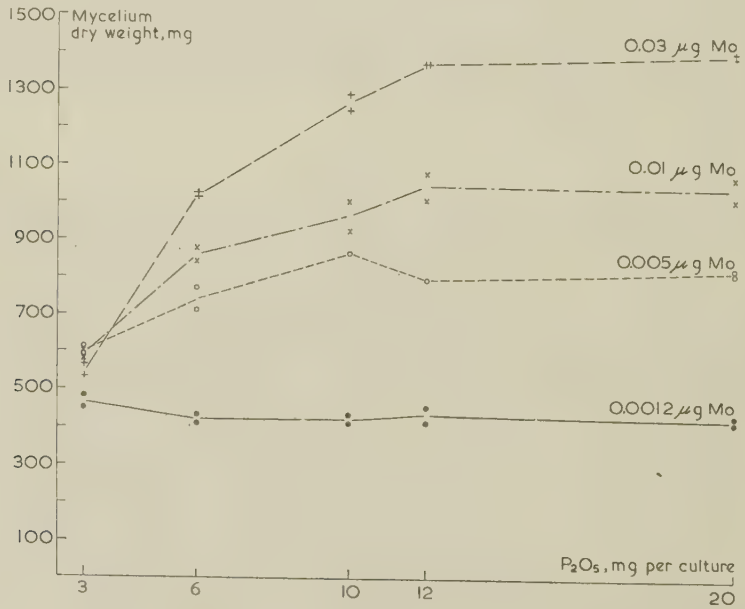

Fig. 3. Effect of phosphate on response of *Aspergillus niger* to molybdenum.

This means that the poor uptake of molybdenum from the phosphorus-deficient soils by the green plants apparently was

not due to the absence of available molybdenum but to difficulties with its uptake. As may be seen from Fig. 3 response to added molybdenum of *Aspergillus niger* is independent of phosphate supply.

In a subsequent experiment the response of cauliflower plants to added molybdenum was studied on various phosphorus-deficient soils supplied with increasing amounts of KH_2PO_4. The soils used in this experiment were of widely differing character; clay as well as sandy and peaty soils were used. The experiment was carried out in Mitscherlich pots containing approximately 5 kg of soil. Each pot was dressed with 3 g of KNO_3 and 0.5 g of $MgSO_4$. After thinning to 5 plants per pot, additional dressings of 3 and 4 g KNO_3 per pot were given. The nutrient salts used were practically free from molybdenum.

Initially a very pronounced response to added molybdenum of the cauliflower plants was observed on practically every soil and at each P-level. The molybdenum-treated plants had a dark green colour as contrasted to the mottled and yellow-green appearance of the no-molybdenum plants. After some time clear differences in degree of molybdenum deficiency were observed between plants dressed with different amounts of phosphate. Plants supplied with inadequate amounts of phosphate showed pronounced symptoms of molybdenum deficiency, those with ample phosphate only slight symptoms. This beneficial effect of added phosphate on molybdenum supply of the plants is in agreement with the results of the above-mentioned experiments.

Plants dressed with 10 or 30 mg of sodium molybdate per pot responded clearly to the phosphate dressings but optimal growth was obtained at a lower phosphate level than was the case with plants growing without added molybdenum (cf Plate VII). This demonstrates that part of the effect of a phosphate dressing on cauliflower plants must be attributed to its influence on molybdenum nutrition. Table XXIV gives the dry weights of the cauliflower plants, a few weeks after the beginning of heart formation. Dry weight of hearts is also given. The latter is more useful as an index for molybdenum supply than total weight of plants. Plants with moderate symptoms of molybdenum deficiency generally have poor hearts but their leaf development may be more luxuriant than is the case with molybdenum-dressed plants.

From the results of this experiment it will be seen that the

TABLE XXIV

Effect of phosphate and molybdate dressings on yield (g per pot) and appearance of cauliflower, grown on phosphorus-deficient soils

Soil: Exp. field and type	KH_2PO_4, g per pot	No added molybdenum *)			+ molybdenum **)		
		Total dry weight	Dry weight of hearts	Appearance †)	Total dry weight	Dry weight of hearts	Appearance
65, sandy	0	0.4	0	—	0.5	—	—
	1	1.5	0	—	0.7	—	—
	3	10.8	0	sev	13.0	0.6	n
	5	15.4	0	sev	12.6	1.6	n
465, peaty clay	0	13.3	0	sev	19.4	0.5	n
	1	21.3	0.9	mod	24.6	2.0	n
	3	18.4	1.5	mod	26.3	3.9	n
	5	26.3	2.0	sl	30.4	6.8	n
962, peaty clay	0	0.7	0	—	0.4	0	—
	1	25.1	1.1	sl	24.7	3.4	n
	3	35.1	7.1	n	36.6	8.0	n
	5	37.8	7.8	n	38.5	8.6	n
1006, clay	0	16.7	0.5	n	12.5	1.3	n
	1	26.9	3.1	n	24.7	2.4	n
	3	22.8	2.5	n	26.0	2.7	n
	5	25.8	5.0	n	27.0	4.2	n
1007, clay	0	3.2	0	—	5.3	0	—
	1	12.4	0.8	n	7.3	0.5	n
	3	16.0	1.6	n	14.6	1.2	n
	5	16.1	2.1	n	13.8	1.0	n
1008, clay	0	3.3	0	—	1.0	0	—
	1	16.7	0.3	n	19.8	0.3	n
	3	22.2	0.9	n	18.9	0.3	n
	5	22.3	0.7	n	21.0	0.7	n
1113, sandy	0	0.4	0	—	0.4	0	—
	1	1.4	0	sev	5.9	0	n
	3	11.3	1.0	sl	19.4	1.3	n
	5	26.0	1.8	n	19.7	0.7	n
1365, sandy	0	0.4	0	—	0.4	0	—
	1	5.9	0	sev	20.3	1.1	n
	3	21.5	0.7	mod	24.9	1.1	n
	5	22.6	0.6	sl	22.3	1.4	n
Ma, sandy	0	14.6	0	sev	33.4	5.5	n
	1	27.3	1.9	mod	35.1	7.2	n
	3	33.5	1.6	sl	31.6	8.5	n
	5	31.7	3.7	sl	31.8	9.4	n
Ho, sandy	0	15.5	0.3	sev	16.9	0.9	n
	1	25.9	1.6	mod	27.7	3.8	n
	3	22.1	1.0	sl	31.8	5.2	n
	5	30.0	4.1	sl	25.8	4.3	n

*) 0 Mo: averages of duplicate values.

**) + Mo: average yields of plants dressed with 10 and 30 mg of $Na_2MoO_4.2H_2O$ per pot, containing approximately 5 kg of soil.

†) sev = severely, mod = moderately, sl = slightly molybdenum-deficient; n = no symptoms of molybdenum deficiency.

Plate VII. Effect of phosphate dressings on molybdenum supply of cauliflower plants grown on phosphorus-deficient soils. A and B: soil 1113, C and D: soil 1365. From *left* to *right*: 1, 3 and 5 g KH_2PO_4 per pot. A and C: no molybdenum added; B and D: 10 mg $Na_2MoO_4.2H_2O$ per pot.

A

B

C

Plate VIII. Effect of nitrogen and molybdenum dressings on tomato plants growing on a molybdenum-deficient soil. A: 0.5, B: 1.0, C: 2.5 g KNO_3 per pot. From *left* to *right*: 0, 50 and 1000 μg $Na_2MoO_4 . 2H_2O$ added per pot.

plants grown on the P-deficient clay soils did not respond to added molybdenum. The slight response which was observed in the young plants disappeared at a later stage. Plants grown on the peaty clay soil from Exp. 962 and particularly those grown on the soil from Exp. 465, which was rich in iron, responded very clearly to added molybdenum. The same is true of plants grown on the P-deficient sandy soils.

In order to know whether the beneficial effect of added phosphate on the molybdenum supply of the cauliflower plants was due to an improved availability of soil molybdenum or to improved uptake of the latter, available molybdenum in the soils was determined with *Aspergillus niger*. Uptake of molybdenum by the fungus is independent of phosphate supply (cf Fig. 3). The results of this experiment are given in Table XXV. It will be seen that in most cases availability of soil molybdenum was enhanced considerably by the phosphate dressings. Soils treated on the field with phosphate had somewhat lower values than those of the pot experiments. This may have been due to the difference in time of application or dose of applied phosphate.

TABLE XXV

Effect of phosphate dressings of phosphorus-deficient soils on available molybdenum, ppm of dry soil, estimated with *Aspergillus niger* *)

Soil	pH	No P added	5 g KH_2PO_4 per pot	Soil from plots dressed with P
Exp. 65, sandy	4.5	0.024	0.033	0.020
„ 465, peaty clay	4.7	0.111	0.118	—
„ 962, peaty clay	4.8	0.207	>0.4	—
„ 1006, clay	5.3	0.208	>0.4	0.350
„ 1007, clay	6.0	0.202	0.249	0.280
„ 1008, clay	6.6	0.057	0.096	0.075
„ 1113, sandy	4.5	0.006	0.022	0.014
„ 1365, sandy	4.8	0.027	0.019	0.024
Ma, sandy	5.7	0.055	0.082	—
Ho, sandy	5.5	0.094	0.106	

*) Averages of duplicate values.

The effect of phosphate on molybdenum uptake by green plants was also studied by Stout *et al.* [46]) and by Barshad [7]). The former grew tomatoes, peas and subterranean clover on a number of acid soils with varying molybdenum content. Symptoms of molybdenum deficiency in the plants were not observed, however, so that the authors derived their conclusions from molybdenum analyses of the plant tissues. Phosphate

dressings were found to enhance the molybdenum content of the plants considerably, particularly when small amounts of molybdate had been added to the soils. In addition to these experiments with soil, culture-solution experiments with tomato plants revealed a highly stimulating effect of phosphate, presumably of $H_2PO_4^-$, on uptake of molybdenum and on its translocation from the roots to the tops.

Barshad [7]) studied the effect of added superphosphate and phosphoric acid on water-soluble molybdenum of a neutral clay loam and on molybdenum content of Ladino clover (*Trifolium repens* var. *latum*) growing on this soil. Although phosphate treatment did not increase the availability of soil molybdenum, uptake of the latter by the clover was clearly enhanced. A beneficial effect of phosphate on molybdenum uptake was also found by Walsh *et al.* [47]). Whether the stimulating effect of phosphate on molybdenum uptake must be attributed to the formation of a complex phosphomolybdate anion which is absorbed more easily by the plant than the molybdate anion alone [7]) or to biochemical processes [46]) remains to be decided.

Effect of copper on response of green plants to molybdenum

Excessive amounts of molybdenum in pasture plants may cause a poisoning of cattle [7]) [9]) [11]) [17]) [47]). Small amounts of copper sulphate

TABLE XXVI

Influence of added copper sulphate on Mo-effect. White clover on molybdenum-deficient soil. Yield, dry weight per pot, g *)

$Na_2MoO_4.2H_2O$	$CuSO_4.5H_2O$, mg per pot						
μg per pot	0	25	50	100	200	500	1000
1st cutting							
0	4.1	3.7	3.4	3.8	3.7	3.3	1.3
25	4.9	5.3	5.3	5.3	5.3	4.4	1.5
50	6.1	6.9	5.8	5.9	6.1	4.9	1.4
100	5.9	5.8	6.3	6.0	5.2	4.5	1.3
500	6.0	6.2	7.0	6.1	5.6	4.9	2.1
1000	5.8	7.0	6.6	6.1	5.8	5.1	1.8
5000	6.3	6.2	6.3	6.6	6.5	5.1	1.8
25000	5.9	6.3	6.3	6.5	6.4	5.4	1.7
2nd cutting							
0	5.0	4.6	4.4	4.7	4.7	3.9	2.3
25	5.1	4.5	4.0	4.8	3.7	4.5	3.8
50	5.3	4.9	4.8	4.1	5.1	4.9	3.8
100	5.4	5.2	4.5	5.0	5.2	4.7	4.1
500	6.0	4.8	5.7	5.0	4.8	4.8	4.2
1000	5.4	5.6	5.2	5.2	5.0	5.0	4.7
5000	5.7	5.3	5.5	4.8	5.2	5.3	4.5
25000	4.5	5.4	5.6	5.7	5.8	5.0	4.0

*) Averages of duplicate values.

supplied to the animals were found to give an alleviation of this molybdenum effect [11] [17] [47].

B u r e m a and W i e r i n g a have demonstrated the existence of a molybdenum-copper relationship in *Azotobacter chroococcum* [10]. With increasing amounts of added molybdenum increasing amounts of copper were found to be required for the formation of the black pigment in ageing colonies of this bacterium.

In order to determine if a similar relationship between molybdenum and copper exists in green plants, a pot experiment with white clover growing on molybdenum-deficient soil at different levels of added molybdenum and copper was carried out. The results of this experiment have been recorded in Table XXVI. It will be seen that over a wide range of concentrations no interaction between Cu and Mo existed. There is an indication, however, that in the absence of added molybdenum, copper sulphate unfavourably affected the development of the plants. Supplied with molybdate, copper sulphate exerted a slightly beneficial effect. It must be stressed, however, that at least part of the effect of copper sulphate could be attributed to its sulphate content.

Effect of various nitrogen compounds on molybdenum requirement of green plants

Without added nitrogen the symptoms of molybdenum deficiency in cauliflower were found to be less pronounced than in the presence of KNO_3. In one experiment, started in the autumn of 1950, the plants without added nitrogen were practically free from Mo-deficiency whereas those growing in the presence of nitrate showed severe deficiency symptoms (Plate VI). In a subsequent experiment the difference between the plants growing without added nitrogen and those with 500 mg of KNO_3 per pot was slight but with 1500 mg of KNO_3 the molybdenum deficiency symptoms were more pronounced (Table XXVII). High amounts of KNO_3 apparently interfere with uptake of molybdenum or with its activity within the plant. In cauliflower this effect is much less pronounced, however, than that of manganese or sulphates. In the case of tomato and sugar beet the nitrate effect was found to be very pronounced (see Plate VIII) whereas only a slight influence of added sulphate on molybdenum uptake was observed.

TABLE XXVII

Effect of KNO_3, NH_4OH and NH_4Cl on yield, g dry weight *) per pot, of cauliflower plants grown on a molybdenum-deficient soil at different amounts of added sodium molybdate

Nitrogen dressing (mg N per pot) and compound	$Na_2MoO_4.2H_2O$, μg per pot					
	0	25	50	100	500	2500
no added N	0.3 (sev) **)	1.1 (sl)	1.0 (n)	1.5 (n)	1.4 (n)	1.6 (n)
500 mg N, KNO_3	0.5 (sev)	1.8 (sl)	2.8 (n)	3.5 (n)	3.9 (n)	3.8 (n)
1500 mg N, KNO_3	0.5 (sev)	0.7 (d)	1.6 (d)	3.1 (n)	4.2 (n)	4.0 (n)
500 mg N, NH_4OH	0.5 (sev)	1.3 (d)	—	—	2.9 (n)	—
500 mg N, NH_4Cl	0.3 (sev)	0.8 (d)	—	—	3.6 (n)	—

*) Averages of duplicate values.

**) sev = severely molybdenum-deficient, d = deficient, sl = slightly deficient, n = normal plants.

The aggravating effect of high nitrate on "whiptail" symptoms of cauliflower was also observed by A g a r w a l a [1]). B a r s h a d [7]) found considerably lower molybdenum values in Ladino clover and in *Lotus corniculatus*, grown on a soil rich in available molybdenum, when dressed heavily with a mixture of $Ca(NO_3)_2$ and NH_4NO_3 than without a nitrogen dressing.

In the case of NH_4OH, NH_4Cl and NH_4NO_3 (see Plate VE) as a nitrogen source the symptoms of molybdenum deficiency in cauliflower were as pronounced as those with nitrate nutrition. The molybdenum-deficient plants supplied with NH_4-N had darker green young leaves, however, than those supplied with nitrate. This may be an indication that chlorophyll formation, *i.e.* protein formation, does not take place in molybdenum-deficient nitrate plants but proceeds normally in ammonium-supplied plants, due to the fact that molybdenum is required for nitrate reduction. Cupping and withering of the leaves of molybdenum-deficient NH_4-plants were similar to those of nitrate plants. In some cases tendency to wilting and withering of the molybdenum-deficient leaves was more pronounced in the plants supplied with ammonium nitrogen than in the nitrate plants. This shows that besides being necessary for nitrate reduction molybdenum is required by cauliflower plants for other reactions. Similar results were obtained in spinach growing on molybdenum-deficient soil. Treated with NH_4NO_3 the colour of the plants was greener than that of the nitrate plants but wilting and withering of large parts of the leaves were more pronounced in the former plants. In the case of tomato

practically no differences in molybdenum-deficiency symptoms were observed between NO_3- and NH_4-treated plants growing on soils of the above-mentioned type. In both cases mottled leaves, upward curling of the leaflets and reduced growth of the plants were the main symptoms.

These results with green plants are more or less in contrast with those obtained with *Aspergillus niger* (see [33]). In that case it was found that with nitrate as the sole nitrogen source dry weight of yield of the 0 Mo-cultures was only 18 per cent of that grown in complete nutrient solution whereas with ammonium sulphate – nitrate the 0 Mo-cultures had produced 67 per cent of the dry weight of similar cultures supplied with molybdenum. With ammonia as the sole N-source a value of 91 per cent was obtained. Although nitrate reduction is not the only function of molybdenum in *Aspergillus niger*, it is undoubtedly, as in denitrifying bacteria [33]) one of its main functions. This conclusion is in agreement with the finding of Nicholas and Nason [39]) that the enzyme which is responsible for nitrate reduction in *Aspergillus* ("nitrate reductase") contains molybdenum as an active component.

In the green plants of the above-mentioned experiments a function other than one related to nitrate assimilation may be of more importance. That in these plants molybdenum is also functioning in nitrate assimilation may be, however, concluded from an experiment with finely cut leaf and stem tissues of cauliflower and tomato plants suspended in solutions with nitrate and malic acid. Nitrate reduction proceeded much more readily in plants supplied with molybdenum than in molybdenum-deficient ones (Table XXVIII). It has not yet been decided whether these results demonstrate the essentiality of molybdenum for "nitrate reductase" or for malic dehydrogenase. The latter may reduce DPN (diphosphopyridine nucleotide) to DPNH which according to Nason and Evans [37]) may be used instead of TPNH for functioning of "nitrate reductase".

In separate experiments, using triphenyltetrazolium chloride as H-acceptor, malic dehydrogenase was found to be more active in cauliflower and bean plants supplied with molybdenum than in molybdenum-deficient plants. Similar results have been obtained by Hewitt and Agarwala [20]) and by Evans *et al.* [16]).

When a comparison was made between plants grown without

TABLE XXVIII

Effect of molybdenum and nitrogen nutrition of cauliflower and tomato plants on nitrate reducing capacity of leaf and stem tissues

Plant tissue	Treatment of plants	μg $NaNO_2$ formed in $\frac{1}{2}$ h at 37°C *)	
		per 1 g dry weight	per 1 mg protein
Cauliflower, leaf	0 Mo + nitrate	42	0.3
,, ,,	low ,, + ,,	293	1.6
,, ,,	normal ,, + ,,	790	3.0
,, ,,	normal ,, 0 ,,	363	1.7
,, stem	0 ,, + ,,	121	1.6
,, ,,	low ,, + ,,	206	3.0
,, ,,	normal ,, + ,,	329	5.9
,, ,,	normal ,, 0 ,,	178	2.5
Tomato, leaf	0 Mo + nitrate	34	0.2
,, ,,	low ,, + ,,	40	0.2
,, ,,	normal ,, + ,,	453	2.0
,, ,,	normal ,, 0 ,,	37	0.3
,, stem	0 ,, + ,,	101	1.5
,, ,,	low ,, + ,,	209	3.4
,, ,,	normal ,, + ,,	387	6.1
,, ,,	normal ,, 0 ,,	158	3.2

*) Anaerobically (Thunberg tubes). The medium used consisted of: 1 g finely cut, fresh plant tissue, 5 cc 0.06 *M* phosphate buffer (pH 7.1), 1 cc 0.1 *M* malic acid, 1 cc 0.1 *M* KNO_3, 2 cc H_2O. Without added nitrate considerably lower nitrite values were found. The same was true in the absence of added malic acid. Quadruplicate values.

added nitrogen but supplied with molybdenum and those supplied with nitrogen but no molybdenum, it appeared that the symptoms of Mo- and N-deficiency in cauliflower were not identical. Although in both cases the plants had chlorotic leaves, molybdenum-deficient plants showed a much more pronounced mottling. Furthermore a severe upward cupping of the leaves occurred which was absent in nitrogen-deficient plants. A further difference was seen in the behaviour of the seed leaves. In the case of nitrogen deficiency the seed leaves completely lost their green colour and subsequently succombed apparently as a result of the translocation of nitrogenous compounds to the growing tissues. In the molybdenum-deficient plants the seed leaves did not loose their dark green appearance notwithstanding the apparent protein deficiency of the chlorotic growing leaves. This could indicate that molybdenum plays some part in break-down of organic nitrogenous compounds or in a translocation reaction.

DISCUSSION

Molybdenum deficiency of green plants is of wide-spread occurrence in the Netherlands and presumably in other countries of Western Europe. This may be concluded from the results of pot and field experiments with leguminous crops, cauliflower, spinach, lettuce etc. recorded in this paper and from a number of recently published papers from other countries [8] [40] [43] [47].

Although the amount of molybdenum required for normal plant growth is extremely small (viz. a few grams per ha per crop) there are a number of soil conditions which depress the availability to or the uptake by plant roots of molybdenum to such an extent that extreme symptoms of molybdenum deficiency may be observed shortly after the plants have developed their first leaves. The following soil conditions have been found to bear some relation to molybdenum-deficient plants: *a*) presence of ironstone, *b*) phosphorus deficiency, *c*) high soil acidity, *d*) excess of manganese, *e*) excess of sulphates.

Excess of iron apparently is an important character of soils on which the plants suffer from pronounced molybdenum deficiency. This may be concluded from the present investigations as well as from a number of reports in the literature. The molybdenum-deficient soils sent to the author from Australia were also rich in ironstone. Iron presumably has a dual effect on the molybdenum supply of the plants, viz. an immobilizing effect on soil molybdenum and a similar effect on soil phosphate. The latter influences molybdenum uptake by the green plants but not that by *Aspergillus niger*. Since the *Aspergillus* values for available molybdenum of ironstone-containing soils in general were found to be extremely low, it is highly probable that a fixation of molybdenum in such soils has taken place. Evidence of such a fixation may be found in Exp. 1094–1 where cauliflower plants showed symptoms of molybdenum deficiency in 1954 on plots dressed in 1951 and 1953 with 4 kg of sodium molybdate per ha, an amount which was sufficient to give normal plants in 1953. Since less than 1% of the applied molybdate has been taken up by the crop in 1953 and leaching out of the soil is improbable, it must be concluded that fixation of the added molybdate had taken place.

Although a close correlation may be expected to exist between

a) and *b*), phosphate in addition to its effect on availability of iron in the soil, has a stimulating influence on uptake and translocation within the plant of available molybdenum. This may be concluded from the work of Stout *et al.* [46]), Barshad [7]) and of the data mentioned in Table XXIII. In the case of *Aspergillus niger* no influence of phosphate on molybdenum uptake was observed (Fig. 3), so that this micro-organism could be used for separating both phosphate effects. In contrast to the results obtained by Barshad who found no increase in water-soluble molybdenum upon treatment of a neutral clay loam with phosphate, available molybdenum of a number of phosphorus-deficient soils was found to be clearly enhanced by phosphate dressings.

High acidity of sandy and peaty soils in a number of cases was found to produce symptoms of molybdenum deficiency in cauliflower. Since available molybdenum as determined with *Aspergillus niger* was relatively high in these soils, interaction with a soil constituent which affects molybdenum uptake by cauliflower but not by *Aspergillus* was assumed. Since molybdenum uptake by cauliflower plants but not that by *Aspergillus* was found to be strongly affected by manganese, it was assumed that high manganese is the factor interfering with molybdenum uptake from acid soils. The values for the absorbed manganese of clover plants grown on these acid soils were not extremely high (Table XXIX) but they were higher than those of most of the samples recorded in Table IV.

TABLE XXIX

Mn, ppm of dry matter, in white clover, grown on acid soils *)					
Exp.	pH	Mn	Exp.	pH	Mn
119	4.1	333	836	3.5	163
10	4.0	95	263	3.7	81
24	4,0	207	837	3.7	207
13	3.9	229	200	4.0	229

*) cf Table XV.

In addition to the influence of high manganese, acidity as such may affect availability of soil molybdenum. Not much difference was found between available molybdenum of deficient soils determined with *Aspergillus niger* at pH 2 of the nutrient solution

and that determined at pH 5 but at pH 6.8 considerably higher amounts were found.

The depressing effect of manganese and of sulphates on molybdenum uptake by cauliflower plants was shown very clearly in pot and field experiments with molybdenum-deficient soils supplied with increasing amounts of molybdate and manganese sulphate, manganese nitrate, manganese citrate and potassium, sodium and calcium sulphates respectively. In culture solutions no such effect of manganese was observed. In the latter case cauliflower plants growing without molybdenum and with low manganese showed more pronounced symptoms of molybdenum deficiency than those grown with moderate or high manganese. Since the response of *Asp. niger* to molybdate was found to be independent of manganese sulphate, the microbiological test was used to see if added manganese sulphate had affected available soil molybdenum. No such effect could be detected, however, so that it has to be assumed that manganese affects in some way the soil-molybdate-root relationship.

Of the plant species studied by the author, cauliflower, spinach, lettuce, tomato and lucerne were very sensitive to molybdenum deficiency when growing on soils of the above-mentioned type. In the case of lucerne nitrogen fixation is primarily affected. It is probable that the sensitiveness to molybdenum deficiency depends on the cooperation of several factors, viz. requirement for molybdenum within the plant tissues, capacity of roots for taking up soil molybdenum, particularly in relation with interfering factors like manganese, sulphate etc. and the size and molybdenum content of seeds. The importance of the latter was shown in experiments with *Phaseolus* beans, peas and wheat, the former two species in pot experiments, the latter in field trials. When commercially purchased seed was used, practically no response to added molybdenum was obtained in bean and peas growing on molybdenum-deficient soil. When seeds of the latter plants were taken, however, severe deficiency symptoms occurred (see Table XXX). In wheat similar differences though less spectacular were obtained. These results are in agreement with those of Wilson [50]), Meagher *et al.*, [29]) and Hewitt and Miles [23]).

The relationship between molybdenum supply and nitrogen metabolism is rather complicated and different for green plants and micro-organisms. In *Aspergillus niger* much higher yields were

TABLE XXX

Effect of molybdenum content of seed on response of *Phaseolus* bean and pea plants to added molybdenum. Yield, g per pot *)

Origin of seed	Year	Mo treatment of plants	Bean		Pea	
			seed	straw	seed	straw
Commercial.	1950	0	13.9	—	10.8	8.3
		+	15.1	—	12.3	9.3
0 Mo plants 1950.	1951	0	6.6	4.3	6.8	7.3
		+	12.9	4.8	8.8	8.6
+ Mo plants 1950.	1951	0	10.3	4.3	8.3	8.3
		+	11.0	4.2	9.3	8.5
0 Mo, 0 seed plants 1951. .	1952	0	3.0	3.3	5.4	4.6
		+	7.7	4.6	7.2	4.6
+ Mo, 0 seed plants 1951. .	1952	0	8.1	4.6	8.1	5.3

*) Averages of duplicate values.

obtained in culture solutions without molybdenum when supplied with ammonium nitrogen than in those supplied with nitrate. This shows that molybdenum plays an important part in nitrate assimilation, a conclusion which is in agreement with the fact that this element has been found to be a constituent of a nitrate-reducing enzyme in *Aspergillus* [39]).

In cauliflower, spinach and tomato growing on molybdenum-deficient soil, however, practically no difference in response to added molybdenum was observed between plants supplied with ammonium nitrate and those with nitrate only. This indicates that retardation of functions other than those concerned with nitrate reduction were responsible for the poor growth and the typical deficiency symptoms of these plants. That the nitrate-reducing capacity of green plants is also adversely affected by molybdenum deficiency, however, may be concluded from the fact that these plants are high in nitrate and low in organic nitrogen, whilst upon addition of molybdenum a ready disappearance of nitrate and formation of organic nitrogen takes place [33]). The experiments with leaf tissues of cauliflower and tomato plants gave also clear evidence as to the effect of molybdenum on nitrate reduction (Table XXVIII). Whether molybdenum affects nitrate reduction directly, as is assumed to be the case in *Aspergillus niger* [39]), or indirectly via a dehydrogenase system is at present undecided. The fact that malic dehydrogenase activity as measured by triphenyltetrazolium chloride was found to be much reduced in

molybdenum-deficient cauliflower and tomato plants may be an indication that the latter conception is right. Also the fact that reduction of selenite to metallic selenium proceeded more readily in macerates of spinach plants supplied adequately with molybdenum than in those of deficient plants. Conclusive evidence that molybdenum affects these reactions specifically and not through a reduction of the protein moiety of the enzymes is lacking, however *).

SUMMARY

A pronounced response to added molybdenum was obtained in leguminous crops (white and red clover, lucerne), cauliflower, cabbage, turnips, radish, spinach, sugar beet, lettuce, tomato, potato and wheat growing on ironstone-containing soils. These soils occur in the Netherlands along brooklets which have their origin in heathy regions. They are often rich in organic matter (low-moor peat), but sometimes they have a more sandy character. Their pH value mostly lies between 5 and 6.

In the leguminous crops reduced fixation of atmospheric nitrogen was the main symptom of molybdenum deficiency. The strongest response to added molybdenum was mostly obtained in the first cutting of these crops. In subsequent cuttings the 0 Mo-crops gradually improved their growth, apparently due to a gradual accumulation of soil molybdenum.

The symptoms of molybdenum deficiency in the non-leguminous crops appeared in a very early stage and mostly in a very severe form. By using a way of planting as described on p. 387 it could be shown that this was due either to fixation of molybdenum in the soil or to the presence of some substance which inhibits uptake of molybdenum by the plant roots.

In addition to these rather clearly defined ironstone soils, molybdenum deficiency was found, particularly in cauliflower, on strongly acid soils of different type: sandy, low-moor, high-moor. Soils of this type were sometimes found to be relatively rich in available molybdenum, as contrasted to the ironstone soils which in general were poor in available molybdenum. For these determinations the *Aspergillus niger* technique was employed. The discrepancy between growth of cauliflower and microbiological test was ascribed to the presence of a high concentration of available manganese in the acid soils which inhibits uptake of molybdenum by cauliflower but not that by *Aspergillus niger*.

The inhibiting effect of manganese on uptake of molybdenum was clearly shown in pot and field experiments with cauliflower plants supplied with increasing amounts of molybdate and of manganese sulphate res-

*) An enzyme which was found to be consistently reduced in molybdenum-deficient tissues was catalase. This was the case whether the activity was calculated per unit of dry matter or per unit of protein. A more detailed account of these biochemical experiments will be given in a separate paper.

pectively. Lettuce reacted similarly to cauliflower though to a somewhat smaller extent, but in the case of spinach, tomato and white clover response to molybdenum was practically unaffected by excessive amounts of manganese.

The antagonizing effect of manganese sulphate on molybdenum uptake by cauliflower was partly due to the manganese ions, partly to the sulphate. This was shown in experiments with various Mn-compounds and with various sulphates, respectively.

A close relationship was found to occur between molybdenum and phosphorus nutrition of green plants growing under natural conditions. Soils on which the plants responded to added molybdenum were found to be poor in available phosphate. In a pot experiment with cauliflower plants phosphate dressings improved molybdenum uptake and growth of plants supplied with very small amounts of added molybdenum. In the case of *Aspergillus niger* no effect of phosphorus sypply on molybdenum uptake was observed.

A pronounced response to added molybdenum was found to occur in cauliflower plants growing on phosphorus-deficient soils from various regions. Dressings of phosphate, without addition of molybdate, on these soils exerted more or less the same effect as a molybdenum dressing. Dressed with molybdenum lower amounts of added phosphate were found to give optimal growth of cauliflower plants than was the case when no molybdenum was added, due to the fact that part of the phosphate effect on cauliflower on these soils was due to an improved molybdenum supply.

A copper-molybdenum relation as has been found to occur in animals and in *Azotobacter chroococcum* was not observed in white clover grown on a molybdenum-deficient soil.

In the absence of added nitrogen, molybdenum-deficiency symptoms of cauliflower, tomato and sugar beet plants were found to be less pronounced than in the presence of high concentrations of nitrate. No difference in response to added molybdenum was found between cauliflower, spinach and tomato plants supplied with ammonium nitrate and those with nitrate only. This is in contrast to the results with *Aspergillus niger* which gave considerably higher yields in the case of NH_4-nitrogen than with nitrates when growing in culture solutions deficient in molybdenum.

Additional evidence was provided that molybdenum plays a part either directly or indirectly in nitrate reduction of green plants.

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REFERENCES

1) Agarwala, S. C., The effect of molybdenum and nitrate status on yield and ascorbic acid content of cauliflower plants in sand culture. Ann. Rept. Agr. and Hort. Research Sta. Long Ashton, Bristol 1950, 83–90.
2) Anderson, A. J., Molybdenum in relation to pasture improvement in South Australia. J. Council Sci. Ind. Research **19**, 1–15 (1946).
3) Anderson, A. J., Thomas, M. P. and Oertel, A. C., Plant responses to molybdenum as a fertilizer. Australia Council Sci. Ind. Research Bull. 198 (1946).
4) Anderson, A. J. and Moye, D. V., Lime and molybdenum in clover development of acid soils. Australian J. Agr. Research **3**, 95–110 (1952).
5) Anderson, A. J. and Spencer, D., Molybdenum in nitrogen metabolism of legumes and non-legumes. Australian J. Sci. Research, Ser. B. **3**, 414–430 (1950).
6) Barshad, I., Molybdenum content of pasture plants in relation to toxicity to cattle. Soil Sci. **66**, 187–195 (1948).
7) Barshad, I., Factors affecting the molybdenum content of pasture plants: II. Effect of soluble phosphates, available nitrogen and soluble sulphates. Soil Sci. **71**, 387–398 (1951).
8) Brandenburg, E., Ernährungsstörungen durch Molybdänmangel bei Blumenkohl. Rheinische Monatsschr. für Gemüse-, Obst- u. Gartenbau No. 4 (1954).
9) Britton, J. W. and Goss, H., Chronic molybdenum poisoning in cattle. J. Am. Vet. Med. Assoc. **108**, 176–178 (1946).
10) Burema, S. J. and Wieringa, K. T., Molybdenum as a growth factor of *Azotobacter chroococcum*. Antonie van Leeuwenhoek **8**, 123–133 (1942).
11) Cunningham, H. M., Brown, J. M. and Edie, A. E., Molybdenum poisoning of cattle in the Swan river valley of Manitoba. Can. J. Agr. Sci. **33**, 254–260 (1953).
12) Davies, E. B., A case of molybdenum deficiency in New Zealand. Nature **156**, 392—393 (1945).
13) Dick, A. T., The effect of inorganic sulphate on the excretion of molybdenum in the sheep. Australian Vet. J. **29**, 18–26 (1953).
14) Evans, H. J. and Nason, A., Pyridine nucleotide-nitrate reductase from extracts of higher plants. Plant Physiol. **28**, 233–254 (1953).
15) Evans, H. J., Purvis, E. R. and Bear, F. E., Colorimetric determination of molybdenum by means of nitric and perchloric acids. Anal. Chem. **22**, 1568—1569 (1950).
16) Evans, H. J., Purvis, E. R. and Bear, F. E., Molybdenum nutrition of alfalfa. Plant Physiol. **25**, 555–566 (1950).
17) Ferguson, W. S., Lewis, A. H. and Watson, S. J., The teart pastures of Somerset. I. The cause and cure of teartness. J. Agr. Sci. **33**, 44–51 (1943).
18) Hannay, J. W. and Street, H. E., Studies on the growth of excised roots. III. The molybdenum and manganese requirement of excised tomato roots. New Phytologist **53**, 68–80 (1954).
19) Hewitt, E. J., Experiments on iron metabolism in plants. I. Some effects of metal-induced iron deficiency. Ann. Rept. Agr. and Hort. Research Sta. Long Ashton, Bristol 1948, 66–80.
20) Hewitt, E. J. and Agarwala, S. C., Reduction of triphenyltetrazolium chloride by plant tissues and its relation to molybdenum status. Nature **169**, 545–547 (1952).
21) Hewitt, E. J. and Bolle-Jones, E. W., Molybdenum as a plant nutrient. I. The influence of molybdenum on the growth of some *Brassica* crops in sand cultures. J. Hort. Sci. **27**, 245–256 (1952).

22) Hewitt, E. J. and Hallas, D. G., The use of *Aspergillus niger* (Van Tiegh.) M strain as a test organism in the study of molybdenum as a plant nutrient. Plant and Soil **3**, 366–408 (1951).
23) Hewitt, E. J. and Miles, P., The effects of seed reserves on the production of deficiencies of copper, zinc and molybdenum in some large-seeded leguminous crops. Ann. Rept. Agr. and Hort. Research Sta. Long Ashton, Bristol 1952, 199–203.
24) Jensen, H. L., Nitrogen fixation in leguminous plants. VII. The nitrogen-fixing capacity of root nodule tissue in *Medicago* and *Trifolium*. Proc. Linnean Soc. N. S. Wales **72**, 265–291 (1948).
25) Johnson, C. M., Pearson, G. A. and Stout, P. R., Molybdenum nutrition of crop plants. II. Plant and soil factors concerned with molybdenum deficiencies in crop plants. Plant and Soil **4**, 178–196 (1952).
26) Kniphorst, L. C. E., Gecombineerde bepaling van het gehalte aan ijzer en mangaan in voedingsmiddelen met behulp van sulfosalicylzuur en formaldoxime. Chem. Weekblad **42**, 311–316, 328–334 (1946).
27) Lewis, A. H., The teart pastures of Somerset. II. Relation between soil and teartness. J. Agr. Sci. **33**, 52–57 (1943).
28) Löhnis, M. P., Manganese toxicity in field and market garden crops. Plant and Soil **3**, 193–222 (1951).
29) Meagher, W. R., Johnson, C. M. and Stout, P. R., Molybdenum requirement of leguminous plants supplied with fixed nitrogen. Plant Physiol. **27**, 223–230 (1952).
30) Millikan, C. R., Effect of molybdenum on the severity of toxicity symptoms in flax induced by an excess of either manganese, zinc, copper, nickel or cobalt in the nutrient solutions. J. Australian Inst. Agr. Sci. **13**, 180–186 (1947).
31) Mitchell, K. J., Preliminary note on the use of ammonium molybdate to control whiptail in cauliflower and broccoli crops. New Zealand J. Sci. Technol. **27**, 287–293 (1945).
32) Mulder, E. G., Over de betekenis van koper voor de groei van planten en microorganismen. Thesis. Wageningen, 1938.
33) Mulder, E. G., Importance of molybdenum in the nitrogen metabolism of microorganisms and higher plants. Plant and Soil **1**, 94–119 (1948).
34) Mulder, E. G., The microbiological estimation of copper, magnesium and molybdenum in soil and plant material. Anal. Chim. Acta **2**, 793–800 (1948).
35) Mulder, E. G., De betekenis van molybdeen voor de plantenvoeding, in het bijzonder in verband met de stikstofvoeding. Landbouwkund. Tijdschr. **62**, 311–318 (1950).
36) Mulder, E. G., Molybdenum in relation to nitrogen fixation of leguminous crops. Trans. Intern. Congr. Soil Sci. (Amsterdam) **2**, 124–126 (1950).
37) Nason, A. and Evans, H. J., Triphosphopyridine nucleotide-nitrate reductase in *Neurospora*. J. Biol. Chem. **202**, 655–673 (1953).
38) Nicholas, D. J. D. and Fielding, A. H., The use of *Aspergillus niger* (M) for the determination of magnesium, zinc, copper and molybdenum available in soils to crop plants. J. Hort. Sci. **26**, 125–147 (1951).
39) Nicholas, D. J. D. and Nason, A., Molybdenum and nitrate reductase. II. Molybdenum as a constituent of nitrate reductase. J. Biol. Chem. **207**, 353–360 (1954).
40) Ødelien, M. and Sorteberg, A., Molybdenum deficiency in lettuce in pot experiments. Inst. of fertilization and soil management. Agr. Coll. of Norway No 18, (1952).

41) Oertel, A. C., Prescott, J. A. and Stephens, C. G., The influence of soil reaction on the availability of molybdenum to subterranean clover. Australian J. Sci. **9**, 27–28 (1946).

42) Piper, C. S. and Beckwith, R. S., A new method for determination of small amounts of molybdenum in plants. J. Soc. Chem. Ind. (London) **67**, 374–379 (1948).

43) Plant, W., The control of "whiptail" in broccoli and cauliflower. J. Hort. Sci. **26**, 109–117 (1951).

44) Romijn, G., De colorimetrische bepaling van het salpeterig zuur. Chem. Weekblad **11**, 1115–1116 (1914).

45) Steinberg, R. A., Role of molybdenum in the utilization of ammonium and nitrate nitrogen by *Aspergillus niger*. J. Agr. Research **55**, 891–902 (1937).

46) Stout, P. R., Meagher, W. R., Pearson, G. A. and Johnson, C. M., Molybdenum nutrition of crop plants. I. The influence of phosphate and sulphate on the absorption of molybdenum from soils and solution cultures. Plant and Soil **3**, 51–87 (1951).

47) Walsh, T., Neenan, M. and O'Moore L. B., The importance of molybdenum in relation to some cropping and livestock problems under Irish conditions. J. Dept. Agr. (Ireland) **48**, 32–43 (1951–'52).

48) Warington, K., Molybdenum as a factor in the nutrition of lettuce. Ann. Appl. Biol. **33**, 249–254 (1946).

49) Warington, K., Some interrelationships between manganese, molybdenum and vanadium in the nutrition of soybean, flax and oats. Ann. Appl. Biol. **38**, 624–641 (1951).

50) Wilson, R. D., Molybdenum in relation to the scald disease of beans. Australian J. Sci. **11**, 209–211 (1949).